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Investigating the Ligand Interactions Between *E. coli* PBP1b, Moenomycin-based Compounds, and Beta-Lactam Compounds

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MSc by Research

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Abstract

Antimicrobial resistance is a growing problem in this era. Resistance to the majority of clinical antibiotics including those of a 'last line of defence' nature has been seen in a number of laboratory and clinical settings. One method aiming at reducing this problem is altering existing antimicrobial compounds, in order to improve pharmacological effects (avoiding resistance mechanisms, improved spectrum of use). Analysis of the interactions between the antimicrobial compounds and their targets can determine whether modifications to current antimicrobials (such as moenomycin A, a glycosyltransferase inhibitor) have altered the mode of action.

ecoPBP1B is a bifunctional glycosyltransferase that could be used as a model for beta lactams and moenomycins, aiding in the design and development of novel antimicrobials based on these families. Moenomycin A has not seen high clinical usage due to poor pharmacokinetics and bioavailability. This project aimed to show whether ecoPBP1b can be used as a model for novel antimicrobials, such as seeing whether Moenomycin A analogues (with cell penetrating peptides to facilitate entry into the bacterial cell) still retain their ability to bind to glycosyltransferases. In addition, this project looked at whether different beta-lactams would significantly alter the conformation of the transpeptidase domain of ecoPBP1b.

Building on previous studies into ecoPBP1b, we managed to successfully produce usable quantities of ecoPBP1b for crystallisation in conjunction with a variety of ligands. Crystals were produced potentially containing complexes including such compounds as AI167-p and AI168-p (Moenomycin A analogues), cefotaxime, cephradine, and a novel beta-lactamase inhibitor (CW-019).

3D structures of ecoPBP1b in complex with AI167 were produced, successfully showing the Moenomycin A analogue bound into the transglycosylase active site. This showed that the addition of peptides to the moenomycin molecule has not interfered with the interactions required for the compound to bind to the transglycosylase domain. In addition, ecoPBP1b was co-crystallised with ampicillin bound to the penicillin binding region, which is consistent with previous studies.

There are issues that need to be addressed with regards to ecoPBP1b before it can be used as a reliable model, especially with beta-lactams. However, the main project goal (using ecoPBP1b for novel MoeA analogues) has been achieved, as well as furthering insights into crystallisation of proteins in general.

“Any true wizard, faced with a sign like 'Do not open this door. Really. We mean it. We're not kidding. Opening this door will mean the end of the universe,' would automatically open the door in order to see what all the fuss is about.”

— *Terry Pratchett (The Last Continent)*

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1. Introduction

1.1. Antimicrobial Crisis

Antibiotics have been widely used as a clinical treatment since the introduction of sulphonamides in 1937 (Davies & Davies, 2010, West & Coburn, 1940). Whilst antibiotics revolutionised modern medicine, and dramatically improved the quality of life for millions around the globe (from allowing the performing of invasive surgical procedures through to the introduction of chemotherapy treatments), the cost of these advancements has not been seen as severe until recently (Brown and Wright, 2016). Many of the antibiotics and antimicrobials discovered in the 20th century have had resistance mechanisms develop in previously susceptible bacterial species, and a large proportion are now no longer in regular use due to widespread resistance to their mode of action (Blair *et al.,* 2014).

The need for novel antimicrobial compounds has been considered a worldwide public health concern for decades now (World Health Organisation, 2014). Antimicrobial resistance has been recorded as early as the 1930s, and one of the most widely used antimicrobial compound groups (penicillins) has had mechanisms of resistance identified from 1940 (Abraham & Chain, 1940; Davies & Davies, 2010). This has led to a severe reduction in the number of functional antimicrobial compounds suitable for commercial and medical use. Reductions in funding has been put towards novel antimicrobial research by pharmaceutical companies in recent years, due to the poor economic return on investments in this area (Payne *et al.,* 2007). As such, few notable discoveries have been made in the last few decades (Arias and Murray, 2015).

1.2. Current Antimicrobials

The majority of antimicrobials that have been licensed for use are derived from bacterial and fungal products (Brown and Wright, 2016). Such natural antibiotic products were discovered

in the so-called 'Golden Age' of antibiotics, between 1940 and 1962 (Singh and Barrett, 2006). These antimicrobial compounds have a variety of targets, as shown by table 1. Beta-lactams inhibit cell wall growth by binding to major peptidoglycan synthesising enzymes, tetracyclines target the 30s subunit of ribosomes and prevent protein synthesis, and quinolones have shown the ability to prevent growth via the inhibition of the DNA synthetic enzymes DNA gyrase and topoisomerase (Cheng *et al*., 2013., Singh and Barrett, 2006). The antibiotics currently in use have been heavily modified, as many of the 1st generation compounds of such antibiotic groups have been shown to have clinical resistance develop after large-scale use. This can be clearly seen in the Beta-lactam families, with the development of the later families of cephalosporins, and methicillins (Singh and Barrett, 2006).

CLASS/SUBCLASS	AREA OF EFFECT	PRIMARY TARGET	WHO CLASSIFIED IMPORTANT MEMBERS
BETA-LACTAMS	Peptidoglycan synthesis	Penicillin Binding proteins	Penicillins (Penicillin G & V, ampicillin, amoxicillin, carbenacillin)
			Cephalosporins (cefotaxime, ceftazidime, cefoperazone)
			Carbapenems (ertapenem, faropenem, impenem)
GLYCOPEPTIDES	Peptidoglycan synthesis	PG amino acid sidechains	ramoplanin, vancomycin, teicoplanin
AMINOGLYCOSIDES	Protein synthesis	30S ribosomal subunit	Amikacin, gentamicin, streptomycin tobramycin
TETRACYCLINES	Protein synthesis	30S ribosomal subunit	Tigecycline
MACROLIDES	Protein synthesis	50S ribosomal subunit	clarithromycin, erythromycin, midecamycin, spiramycin
STREPTOGRAMINS	Protein synthesis	50S ribosomal subunit	Quinupristin/dalfopristin, pristinamycin
RIFAMYCINS	RNA synthesis	RNA polymerase	Rifabutin, rifampin, rifaximin
QUINOLONES	DNA synthesis	Topoisomerase II & IV	Cinoxacin, ciprofloxacin, enoxacin, moxifloxacin, pipemidic acid
POLYMYXINS	Cell Membrane	Lipopolysaccharides	Polymyxin B & E

Table 1. Antibiotics classified as critical or highly important by the WHO. Whilst many target similar metabolic pathways, the is significant diversity in the targeted proteins or substrates (Collignon et al., 2009)

Due to the reliance decades (Brown and on these established families of antibiotics, research into novel targets has been lacking in recent Wright, 2016). Whilst novel approaches in recent years have been developed, such as genomic exploitation (looking at highly conserved genetic sequences for potential targets) and the development of the iChip device (able to grow and extract products from previously unculturable bacterial species), they have not been able to repeat the successes of the Golden Era (Nichols *et al.,* 2010). Payne *et al*. (2007) suspect that this is due to the withdrawal of funding towards novel compounds, with larger pharmaceutical companies preferring to develop existing compounds with a higher chance of licensing success than researching untested compounds.

As mentioned earlier, antibiotics target particular areas of metabolic pathways. Improving the understanding of these pathways would potentially open up new areas of antibiotic development. One essential pathway which is universal to bacterial species is the synthesis and modification of peptidoglycan.

1.3. Peptidoglycan synthesis

Some groups of antimicrobials have targets involved in the peptidoglycan synthesis pathways. This is primarily due to the fact that this process is essential to the growth and survival of bacterial cells and, in theory, cannot be dramatically modified easily. Peptidoglycan synthesis is carried out in both Gram-positive and Gram-negative bacteria, but results in different variations of peptidoglycan (Brown *et al*., 2015). Gram-positive bacteria produce multiple layers of peptidoglycan, which form their cell wall, whilst Gram-negative bacteria only have a single layer of peptidoglycan (Popham and Young, 2003).

The structure of peptidoglycan is formed with crosslinked chains of GlcNAc-MurNAc repeating disaccharide units. Much of the formation of peptidoglycan is facilitated by glycosyltransferase and transpeptidase enzymes (Figure 1), which catalyse the formation of the glycan chains and the production of interpeptide bridges respectively (Lovering, Gretes

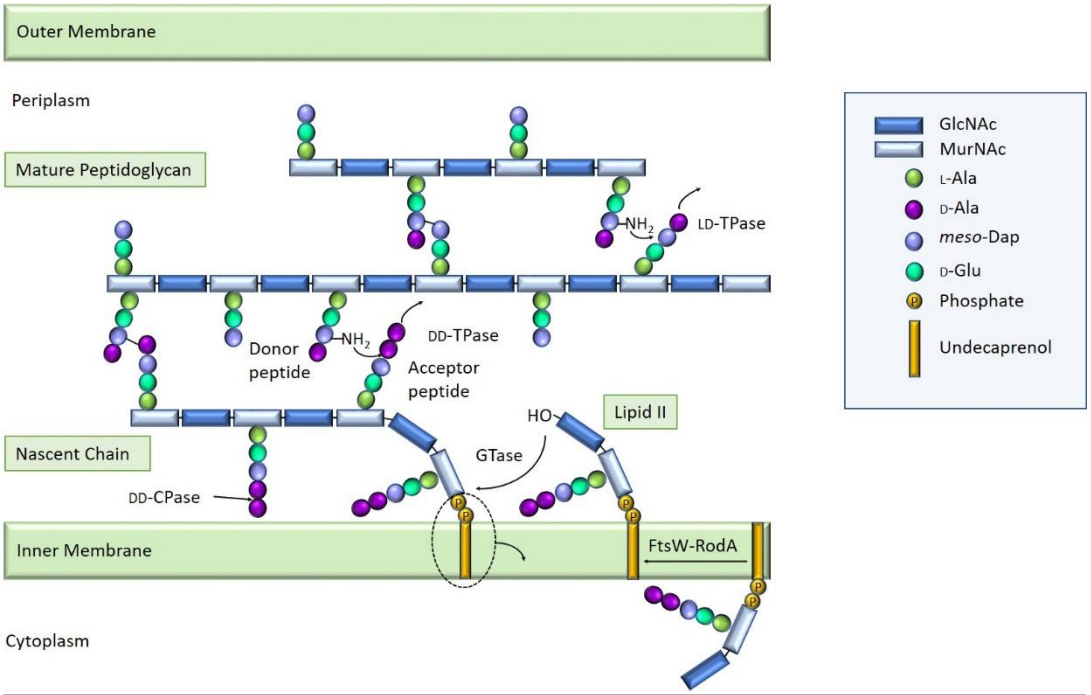

Figure 1. Peptidoglycan structure and Synthesis. Lipid II (a disaccharide-pentapeptide monomer with a bactoprenol-pyrophosphate anchor) is transported to the periplasm where it binds to a glycosyltransferase (GTase). The GTase utilises Lipid II to form a nascent chain (immature peptidoglycan). Sidechains branching from the MurNAc blocks of the nascent chain are used to form the crosslinks to other chains, utilising transpeptidases (TPase). Moenomcyins inhibit the GTases, Beta-lactams inhibit the Tpases.

and Strynadka, 2008). This synthesis occurs in the Gram negative periplasmic space, located between the inner and outer membranes (Typas *et al.* 2011). Glycosyltransferases and transpeptidases are found anchored to the inner membrane (all late-stage peptidoglycan synthases contain transmembrane anchors), where they can encounter their respective substrates. For glycosyltransferases, Lipid II, which is anchored to the inner membrane via a phosphate-undecaprenol molecule; for transpeptidases, the amino acid sidechains presented on the nascent chain (Egan and Vollmer, 2012). Glycosyltransferases facilitate the production of a nascent chain, also called immature peptidoglycan. Transpeptidases (in peptidoglycan synthesis) facilitate the formation of crosslinkages between strands of peptidoglycan. Enzymes with a transpeptidase domain are labelled penicillin binding proteins (PBPs), due to their ability to covalently bind penicillins (Vollmer and Bertsche,

2008). Glycosyltransferases and transpeptidases can be found in monofunctional forms, or bound together, which produces a bifunctional enzyme (Lovering, Gretes and Strynadka, 2008). One major bifunctional enzyme is PBP1b, which contains both a transglycosylase and transpeptidase domain.

1.4. Glycosyltransferases

Glycosyltransferases are defined as enzymes that catalyse the formation of glycosidic bonds via the transfer of sugar moieties from donor molecules to acceptor molecules (Sinnott, 1990). There are currently 103 defined subfamilies of glycosyltransferases according to CAZy, the carbohydrate enzyme database (Cazy.org, 2018). It should be noted that the International Union of Biochemistry and Molecular Biology (IUBMB) has not officially designated subfamilies in glycosyltransferases based on intrinsic structural features, or those which act on distinctive substrates. These can be broadly divided into two groups based on action; inverting and retaining (Sinnott, 1990). Inverting glycosyltransferases have a similar action to inverting glycosidases, in which they invert the anomeric configuration, making glycosidic bonds between sugar molecules of opposite stereochemistry (e.g. UDP-glucose to

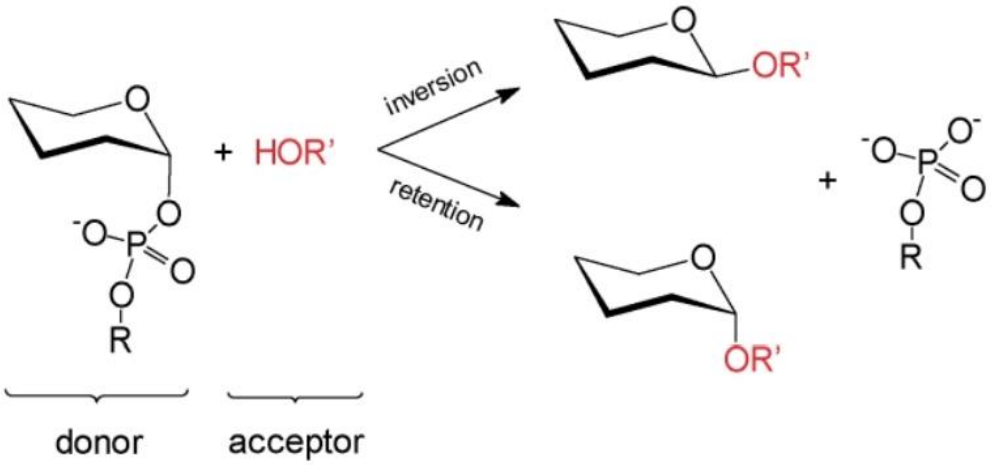

Figure 2. Demonstration of the differences between inverting and retaining glycosyltransferases. The sections highlighted in red represent the sugar donor molecules, which the glycosyltransferases utilise in the phosphate removal (in the case of peptidoglycan synthesis Note the variation between the reactive oxygen groups of the acceptor molecules. Image reproduced from Coutinho *et al.* (2003).

Beta-glucoside). Retaining glycosyltransferases form glycosidic bonds between sugar donors and acceptors with the same stereochemistry (figure 2).

Peptidoglycan-synthesising Glycosyltransferases are expected to have a three-dimensional fold around the active site, which enable the production of polymer chains, such as the nascent chain produced during peptidoglycan synthesis (Gloster, 2014). To date, 3 structurally different folds have been identified; GT-A and GT-B, alongside a third fold found in peptidoglycan-modifying enzymes tentatively labelled GT-C (Lovering, Gretes and Strynadka, 2008). The GT-A fold (characterised with an α/β/α sandwich, resembling a Rossmann fold) and the GT-B fold (consisting of 2 Rossmann domains with a linker region) are not conserved to glycosyltransferases, having been seen in other enzyme families such as sugar epimerases (Campbell *et al.*, 2000; Breton *et al.*, 2005). GT-C folds contain 8-13

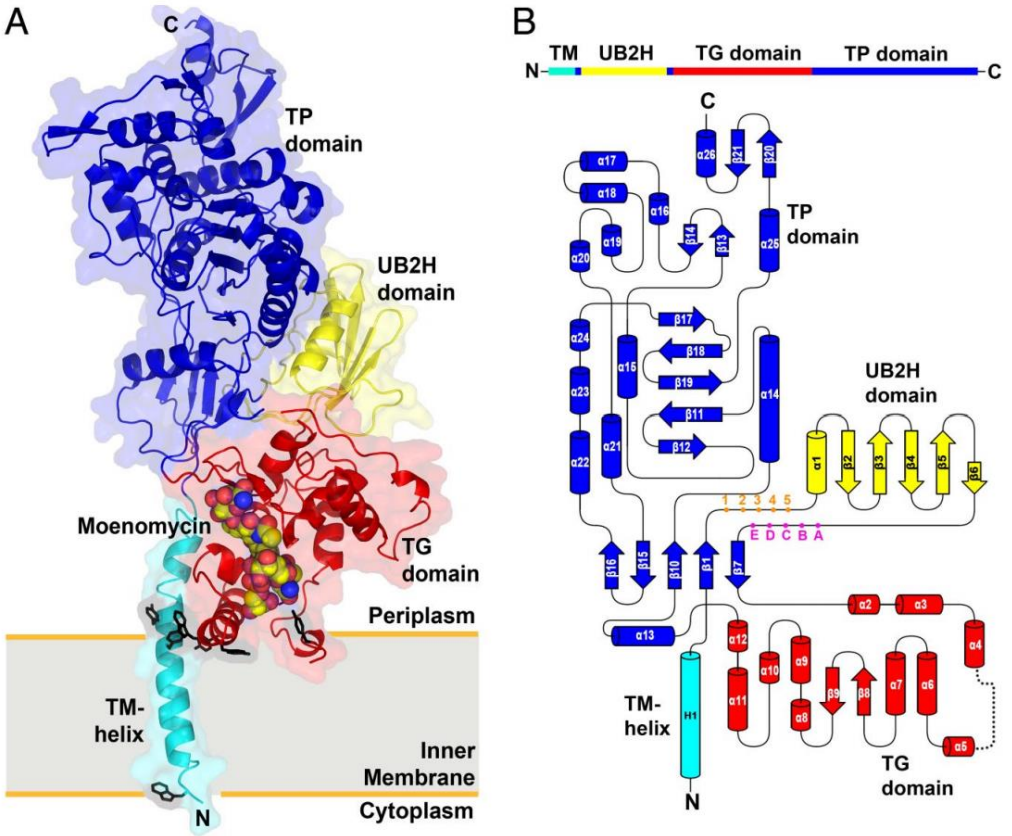

Figure 3. Representations of ecoPBP1b, as characterised by Sung *et al.* (2009). A; Cartoon representation of the 3D structure, with the 4 major domains (TP: transpeptidase, TG: transglycosylase, TM: transmembrane, UB2H) highlighted, as well as the position in the cell. B; 2D structure showing α helices and β sheets, and their relevant domains.

transmembrane helices, and a dependency for lipid-based phosphate-activated donor sugar substrates (Albesa-Jove et al., 2013). Glycosyltransferases identified with a GT-C fold to date have all been inverting enzymes (Lairson et al., 2008). Glycosyltransferases can be monofunctional, where they have a singular transglycosidic function, or bifunctional (with additional domains containing other active sites) (Wang *et al.*, 2001). One major GT-A bifunctional glycosyltransferase is ecoPBP1b which, as mentioned previously, also contains a transpeptidase domain, allowing two concurrent processes required in peptidoglycan synthesis (formation of nascent chains and crosslinkage of such chains) (Sung *et al.*, 2009).

1.5. Transpeptidases

Transpeptidases are involved in late stage peptidoglycan synthesis. They catalyse the peptide bond formation between the D-ala (4) residue and Lys (3) residue located on protruding stem peptides of nascent and mature peptidoglycan, which crosslink between the peptidoglycan chains (figure 4). This reaction results in the removal of the D-ala molecule from the end of a protruding MurNac stem peptide (Lupoli et al., 2011). They are membrane bound and are often closely linked with glycosyltransferases, working on the product of glycosyltransferases (the aforementioned nascent chain) (Egan and Vollmer, 2012). The transpeptidases were first discovered shortly after penicillin, as many were characterised during studies determining the mechanism of action of penicillin (Vollmer and Bertsche, 2008). As such, many are labelled as penicillin binding proteins (PBPs). The transpeptidases are essential for the growth and survival of the bacterial cell, and have been an ideal target for antimicrobials due to their highly conserved nature across bacterial species.

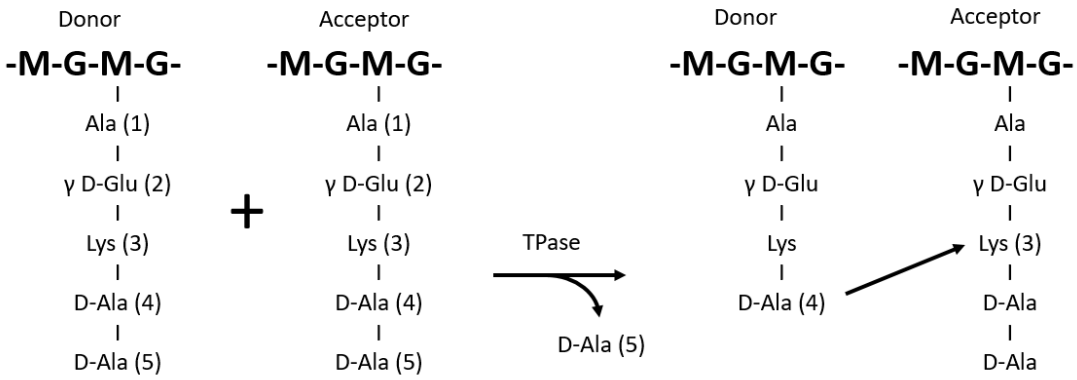

Figure 4. Schematic showing an example of an action of transpeptidases. Transpeptidases utilise the MurNac amino acid sidechains to form the peptide crosslinkage in mature peptidoglycan. In forming the crosslinkages, the terminal D-Ala is removed. It should be noted that there are other forms of transpeptidase action, and this particular action is one carried out by the transpeptidase of this study.

1.6. *E. coli* PBP1b

ecoPBP1b is, as mentioned earlier, a bifunctional glycosyltransferase from family 51. It consists of 4 overall domains; a transmembrane domain (TM), a glycosyltransferase domain (or transglycosylase, TG), a transpeptidase domain (TP) and a UB2H domain (see Figure 3). The TM domain ensures that the overall protein is anchored to the cellular membrane as well as containing a hydrophobic region suspected to enable binding of substrates to the TG domain. The TG domain produces a nascent chain from Lipid II subunits suitable for peptidoglycan synthesis, which is then fed into the TP domain for crosslinking with either mature peptidoglycan or other nascent chains (figure 5). The UB2H domain function hasn't been fully ascertained, but is believed to interact with binding partners for the TP domain (King, Lameignere and Strynadka, 2014).

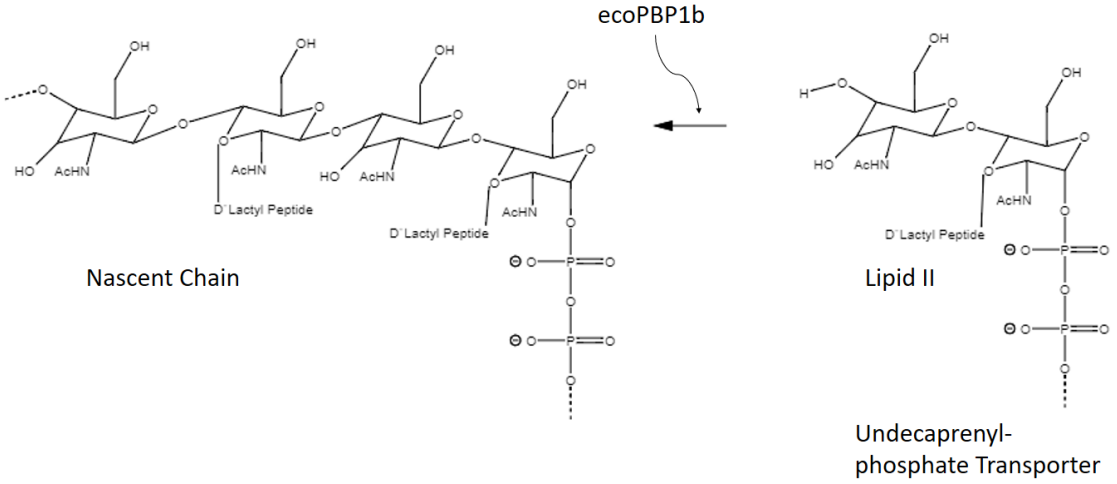

Figure 5. Mechanism of action of ecoPBP1b to elongate the nascent chain, utilising lipid II. The Glc-Nac 4-OH is deprotonated by the TG domain, which directly attacks the C1 of the nascent chain-linked MurNac structure.

Due to the bifunctional nature of ecoPBP1b, it is affected by 2 major classes of antibiotics; beta-lactams and moenomycins. Beta-lactams bind to the penicillin binding region located on the transpeptidase domain, preventing the crosslinkage step of peptidoglycan synthesis. Moenomycins mimic the product produced by ecoPBP1b (including a lipid tail, necessary for the entry to the active site), irreversibly binding to the TG active site.

1.7. Notable Previous Studies of ecoPBP1B

It has been known that ecoPBP1b is involved in peptidoglycan synthesis since the 1970s (Nakagawà, Tamaki and Matsuhashi, (1979); Suzuki, Nishimura and Hirota, (1978)). Several studies have focussed on the glycosyltransferase domain, and how moenomycin A binds to it, in particular Sung *et al.* (2009). This study characterised ecoPBP1b with moenomycin A bound to the TG active site at a resolution of 2.16Å, which produced further insight into the functional ability of ecoPBP1b (Figure 3). This study was notable for being one of the first studies to characterise the entirety of ecoPBP1b (with all 4 domains).

An issue with the Sung *et al*. (2009) characterisation was the potential ambiguity of structural residues, such as the exact locations of residues in the region of H240-T267 (highlighted by King *et al*. (2016)) within the electron density map produced by this study. This allowed for some degree of interpretation to be involved. In addition, it appears that they characterised ecoPBP1b with no stabilising beta-lactam such as ampicillin. Whilst there are imperfections with this study, Sung *et al.* (2009) did provide a basis for further expression and characterisation of ecoPBP1b, much of which was utilised in this research project.

A major problem with characterising the exact mode of action that is carried out by ecoPBP1b (and other monofunctional glycosyltransferases) is the current lack of Lipid II in enough quantity to test (Cheng *et al.*, 2008). There have been some studies attempting to produce synthetic of variations of Lipid II (such as a truncated version which is expected to contain the substrate activity). However, the quantities produced are currently insufficient for extensive studies.

Another key study was carried out by King *et al.* (2016), in which they carried out studies comparable to this project: looking at the binding of moenomycin to the TG domain, as well as binding a number of beta-lactam-based substrates such as ampicillin, cephalexin and CENTA. CENTA is a chromogenic cephalosporin which can be used to crudely identify the presence of various beta-lactamases (Bebrone et al., 2001). They found that the conformation of the active site largely remains unchanged when substrates are bound, with the exception of the side chain at S510 (the catalytic residue), which was believed to position the oxygen of the beta-lactam structure into the oxyanion hole.

This implies that the majority of changes made to the beta-lactam structure for antimicrobials is not for altering or changing binding affinity, but for improving the

pharmacological effects (bioavailability, broadness of action, avoiding resistance, for example).

In order to respond to the antimicrobial crisis, we need to develop novel compounds. Development of such compounds requires an improved understanding of the interactions that occur between current (and novel) compounds and their targeted active site. If these interactions can be elucidated, design of these compounds can be aimed towards overall improvements (decreasing likelihood of resistance mechanisms developing) or specific qualities, such as beta-lactamase resistance or better pharmacological properties.

1.8. Aims and Objectives

It was proposed that ecoPBP1B can be used to elucidate the binding of novel antimicrobial compounds based in 2 antibiotic classes; beta-lactams and moenomycins. Previous studies have looked at using the singular domains for study, as opposed to using the whole model. Using novel moenomycin analogues produced at the University of Lincoln, in conjunction with a range of clinically approved beta-lactams, structures of ecoPBP1B in complex with these compounds were produced.

The aims were twofold; Firstly, to examine the binding of the MoeA analogues, looking at whether the alterations to the MoeA structure have affected binding affinity. Secondly, to examine the conformational changes caused by the binding of beta-lactams to the penicillin binding site, which may show how the different side chains of such compounds affect the TP domain. This was achieved by using *E. coli* to produce quantities of ecoPBP1b to be purified and crystallised in conjunction with the aforementioned ligands.

Aims:

Primary Aim: To define the interactions between novel moenomycin A analogues AI167-p and AI168-p in the glycosyltransferase domain of ecoPBP1b.

Secondary Aim: To model the effect that differing beta-lactam based compound cause on the transpeptidase domain of ecoPBP1b.

2. Moenomycin

2.1. Introduction

Moenomycin A (MoeA) is an important compound in the development of effective counters to antimicrobial resistance. MoeA, a member of the moenomycins family of antimicrobials, is a naturally occurring phosphoglycolipid able to interfere with the synthesis of peptidoglycan (Van Heijenoort *et al.*, 1987). The structure of MoeA was identified by Welzel *et al.* (1981, 1983), but has yet to see wide clinical applications. Its primary use to date has been as a growth promoter in animal feeds, under the names flavomycin and bambermycin (Halliday et al., 2006). What makes Moenomycin A notable is that whilst it has been used for decades as a growth promoter, environmental resistance has been shown to be negligible in *E. faecalis* and *S. aureus*, both of which are species known to develop rapid resistance (Butaye, Devriese and Haesebrouck, 2001, White et al., 2003).

2.1.1. Mode of Action of Moenomycin A

Moenomycin A targets transglycosylase enzymes involved in the formation of the nascent chain required for peptidoglycan synthesis, notably those in family 51. This area of peptidoglycan synthesis has been largely overlooked until recently in favour of the transpeptidase classes of enzymes, targeted by Beta-lactams (Halliday et al., 2006). This could be primarily due to the membrane-bound nature of transglycosylases, which makes

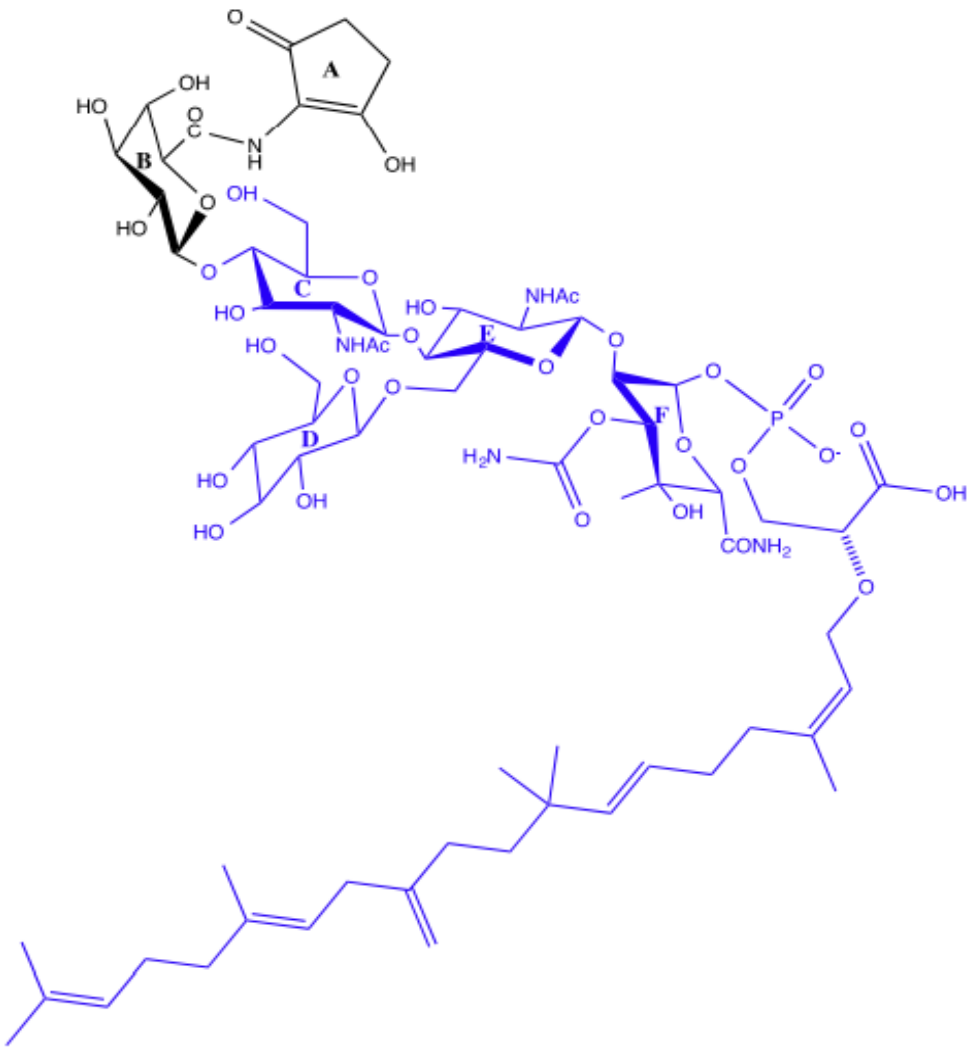

Figure 6. The structure of Moenomycin A, a known potent inhibitor for bacterial transglycosylases. The region highlighted in blue is the minimal inhibitory pharmacophore, which is often used as a scaffold for the design of new potential inhibitors. The sugar units C, E & F are the minimal units required for antibacterial activity. E & F still inhibit TG function, but lack antibacterial activity. Units A, B and D have no impact on antibacterial activity (reproduced from Galley, O'Reilly and Roper (2014).

them significantly harder to study than the transpeptidase class of enzymes (Lovering, Gretes and Strynadka, 2008).

Moenomycin A has been shown to bind reversibly to *E. coli* PBP1B and 1C, as well as the homologous transglycosylases found in *Staphylococcus aureus* (Di Guilmi *et al.* 2003, 2003). Initially hypothesised to compete for the active site against the naturally occurring substrate Lipid II, it is now known that it competes with the growing nascent chain produced by the TG domain, preventing the addition of further Lipid II molecules. It does this by mimicking the

disaccharide–pyrophosphate–prenol linkage naturally formed between the transglycosylase domain and Lipid II (Zuegg et al., 2015).

2.1.2. Structural Characteristics of Moenomycin A

Its ability to inhibit transglycosylases is predominantly down to 2 major characteristics; the binding of the F-ring and phosphoglycerate sections to 6 highly conserved residues (E83, S116, Q121, K124, R132, K137, as identified in *Aquifex aeolicus* PBP1A) located in the transglycosylase active site (Yuan et al., 2008), and the lipid tail, which allows access to the TG active side buried within the inner membrane of the cell. In addition, it has been noted that the lipid tail interacts with the transmembrane domain, demonstrated by Cheng *et al.*

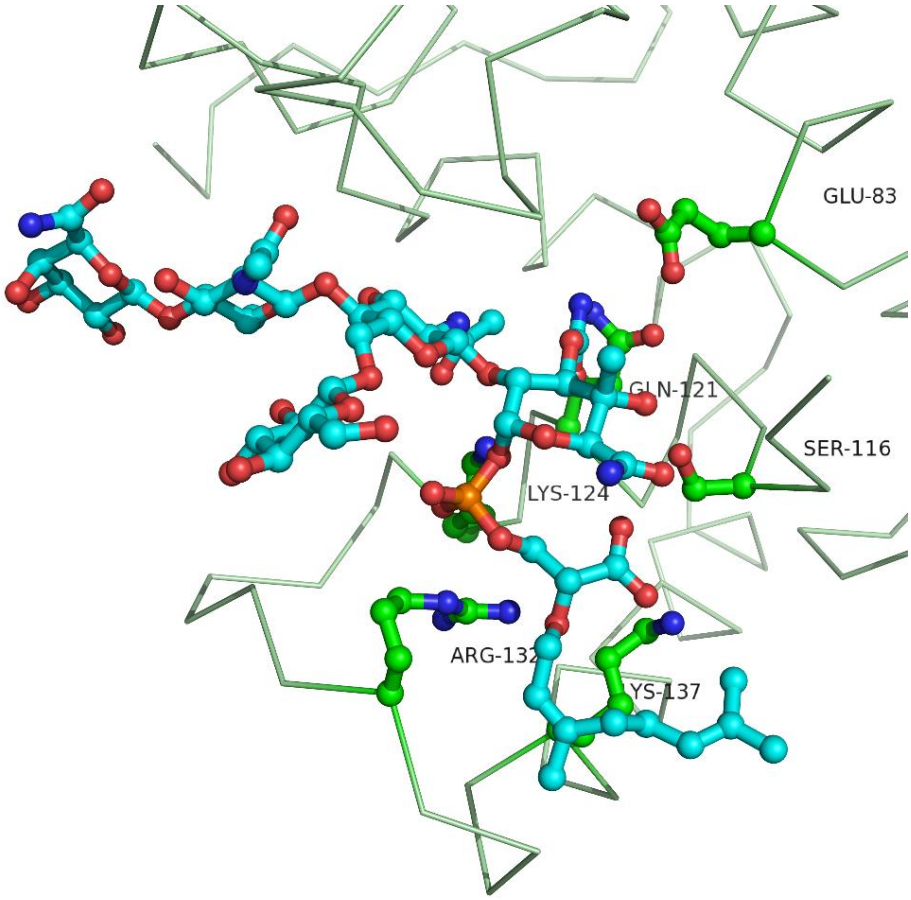

Figure 7. 3D representation of Moenomycin A bound to the active site of 3D3H, a PBP1a found in *A. aeolicus*. Highlighted in green are the 6 residues that Yuan *et al.* (2008) identified as binding to the F ring and phosphoglycerate sections. These identified residues were used in conjunction with subsequent PBP1b models to identify relevant catalytic residues.

(2008) who removed the transmembrane domain of a PBP1B, which then showed a five-fold decrease in the binding affinity of MoeA. This TM-MoeA interaction is most likely due to a 'hydrophobic patch' on the TM domain, which is hypothesised by Yuan *et al.* (2007) to act as a recognition site for binding substrates such as Lipid II.

2.1.3. Effectiveness of Treatments

Whilst MoeA has been shown to have good activity against Gram positive bacterial species, it has a reduced impact when tested on Gram negatives. Moenomycin A is believed to enter the cell via passive diffusion, as opposed to active transport (transport proteins or influx pumps), based on its increased effectiveness on cells with increased cell wall permeability (Ostash and Walker, 2010). Due to the nature of the Gram negative outer membrane, the

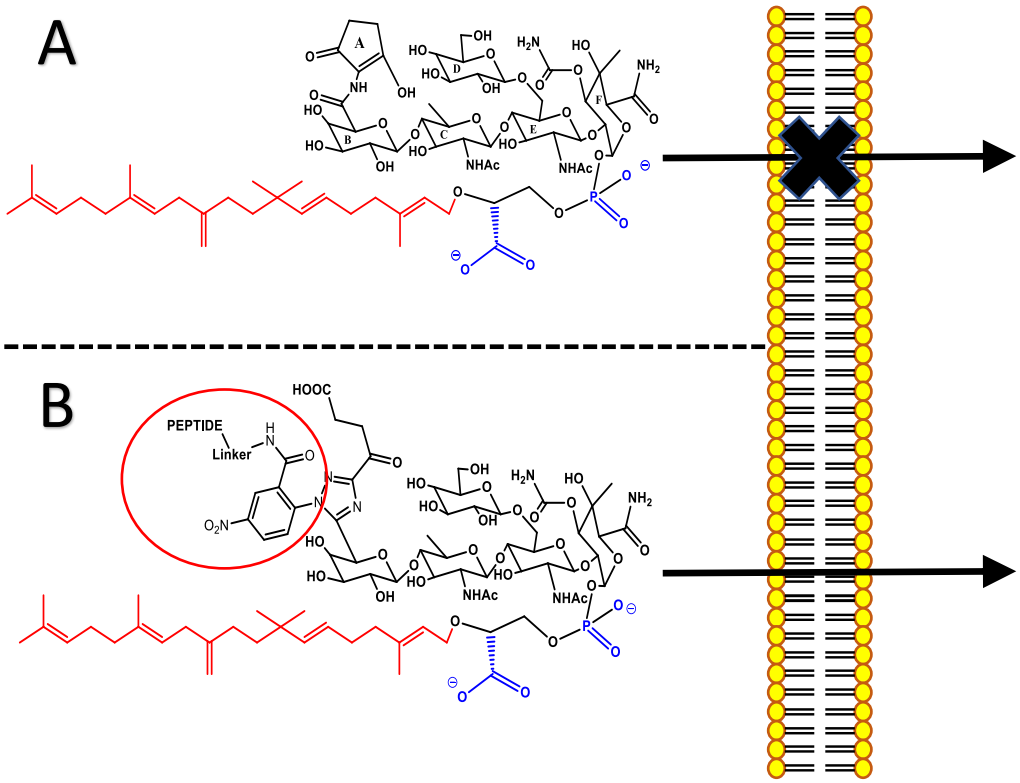

Figure 8. Demonstration of the inability of MoeA to pass through outer membranes. A; MoeA unable to pass through the phospholipid bilayer, due to the carboxylate and phosphate groups (highlighted in blue). B; MoeA with positively charged cell-penetrating peptide and linker attached (circled in red), able to pass through the outer membrane and access the periplasm.

presence of negatively charged carboxylate and phosphate groups reduces moenomycin's ability to enter the negatively charged environment of the cell (Halliday et al., 2006). This, combined with a reduced bioavailability in the bloodstream caused by the hydrophobic lipid tail (necessary for the binding of MoeA to both the TM and TG domains) is one of the major factors why Moenomycin A, and by the same distinction the other moenomycins, has not been used in a clinical setting. Unfortunately, these issues cannot be resolved purely by truncating the lipid tail, as doing so will most likely prevent any analogues from accessing the TG active site. The addition of cell-penetrating peptides may be able to overcome such difficulties (figure 8). Many such peptides are comprised of positively charged residues, in order to attract compounds to the negatively charged outer membranes of bacterial cells and overcome the effects of carboxyl and phosphate groups.

2.1.4. Recent Developments

Moenomycin A has had somewhat of a resurgence in recent years, due to the recent current focus on antimicrobials resistance and the need to develop novel compounds. A large portion of the research has been to alter both the bioavailability, and to broaden its treatments applications (predominantly, targeting Gram Negatives). A notable fact to emerge in the last 25 years regarding MoeA is that fragments of MoeA still demonstrate antimicrobial effects. This produced a study by Sofia *et al.* in 1999, in which the pharmacophore (the sections of a molecule which have pharmacological effects) was identified by comparing 1300 analogues of MoeA at critical binding points to transglycosylases. This pharmacophore was compiled by Galley, O'Reilly and Roper (2014).

2.1.5. Preliminary Findings

Previous research carried out as part of the University of Lincoln Drug Design and Delivery Group has been looking into alterations to moenomycin A, aiming to improve the bioavailability of the compound, in addition to improving its treatment efficacy against

clinically important Gram Negative pathogenic bacterial species (*Acinetobacter baumanii, Klebsiella pneumoniae, Pseudomonas aeruginosa*). MIC testing of altered compounds such as AI167 and AI168 showed marked decreases in the quantities required to inhibit growth (table 2). This has provided a basis for further study into the mode of action of peptide-bound MoeA compounds.

Compound	***P. aeruginosa* ATCC 27853**	***K. pneumoniae* ATCC 700603**	***A. baumannii* ATCC 19606**
	MIC (µg/ml)	**MIC (µg/ml)**	**MIC (µg/ml)**
Moenomycin A	256	64	4
AI167-p	16	4	64
	16	8	64
	16	4	64
AI168-p	32	32	128
	16	32	128
	16	32	128
	MBC (µg/ml)	**MBC (µg/ml)**	**MBC (µg/ml)**
AI167-p	256	256	128
	256	256	256
	256	256	128
AI168-p	256	64	128
	256	128	256
	256	128	256

Table 2. Antimicrobial efficacy of the two moenomycin A analogues AI167-p and AI168-p as compared to the original compound. As can be seen, the two analogues perform better against *P. aeruginosa and K. pneumoniae*.

2.1.6. Research Aims

This chapter of research primarily covers one facet of the rational design approach; testing the binding efficacy of Moenomycin A analogues to a predetermined target: *E. coli* PBP1B. These compounds have had cell-penetrating peptide additions made to the A- ring located opposite the lipid tail (A subunit), as this has been identified in previous studies not to interfere with the binding of MoeA to transglycosylase functions.

The bifunctional glycosyltransferase *E. coli* penicillin binding protein 1b (also known as ecoPBP1b) was chosen to model the MoeA analogues. Its bifunctional nature was utilised in a later chapter of this project as well, looking at another class of antimicrobial compounds.

Specifically, this chapter looked at two major question: What is the nature of the active site binding of the new MoeA-peptide conjugates; and have the increase in MICs observed caused by new interactions with the transglycosylase binding?

In order to answer these questions, structural biological techniques will be utilised to determine any changes in the binding to the TG domain.

2.2. Methods

2.2.1. General Methods

2.2.1.1. Plasmid DNA Miniprep

Bacterial plasmids containing the entire ecoPBP1B gene (TM, TP, UB2H, TG) were purified from XL1-Blue supercompetent *E. coli* cells with the use of the QIAprep Spin miniprep kit (Qiagen). All the reagents and buffers (with the exception of the LB broth) came as part of the aforementioned kit. Single colonies from an XL1-Blue transformation were selected and grown in 5ml LB broth (Melford) cultures with ampicillin (50µg/ml) overnight. These cultures were pelleted at 8000rpm for 3 minutes in 2ml microcentrifuge tubes, and the protocol provided by Qiagen was followed to purify the plasmid samples (QIAprep® Miniprep Handbook, 2017). Purified plasmid samples were then stored at -20^{o}C until use.

2.2.1.2. Calcium Competent cell production

Competent BL21 (DE3) pLysS *E. coli* cells were produced for plasmid transformation and ecoPBP1b expression.

Colonies of BL21 cells were streakplated on LB agar from glycerol stocks, and incubated at 37^{o}C overnight. Single colonies from these plates were used to inoculate 5ml LB broth cultures, and left to grow overnight in an orbital shaker at 37^{o}C, 180rpm. 0.25ml of the 5ml broth culture was used to inoculate a 50ml LB broth culture, and grown at 37^{o}C to an OD_{600nm} of 0.2. Once the OD_{600nm} was achieved, the cultures were cooled on ice.

Cultures were centrifuged at 3000g for 10 minutes, and the supernatant subsequently removed. Pelleted cells were resuspended in 5ml of ice-cold $MgCL_2$ (0.1M), left to stand for 5 minutes, and centrifuged at 3000g for 5 minutes. Once the supernatant had been removed, the pelleted cells were resuspended in 2.5ml of ice-cold $CaCl_2$ (0.1M), and left to stand for 5 minutes. After centrifuging at 3000g for 5 minutes, the supernatant was removed and the

pellet resuspended in 0.5ml of ice-cold $CaCl_2$ (0.1M). The resuspension was kept on ice for a minimum of 90 minutes, before adding glycerol (to a final percentage of 15), and being aliquoted out in 100µl batches. The aliquots were flash frozen in liquid nitrogen and stored at -80^{o}C until use.

2.2.2. Expression and Characterisation of ecoPBP1b

2.2.2.1. Transformation of Expression Cells

100µl *of E. coli* BL21 (DE3) pLysS cells were defrosted on ice after storage at -80^{o}C, and 3µl of plasmid DNA (containing pET15b plasmids with the ecoPBP1b insert) added. The cells were incubated on ice for 30 minutes after DNA addition. Once incubated, the cells were heat shocked at 42^{o}C for 45 seconds, before being cooled on ice immediately afterwards for 1-2 minutes. 250µl of SOC media (2% w/v tryptone, 0.5% w/v Yeast extract, 10mM NaCl, 2.5mM KCl, 20mM glucose) was added, before the transformed cells were incubated at 37^{o}C for 60 minutes. After incubation, 100µl of the cell culture was spreadplated on LB agar with added ampicillin (50µg/ml), and grown overnight at 37^{o}C. Chloramphenicol was not used to retain the PLysS gene during this research.

2.2.2.2. Expression of ecoPBP1b

4-7 colonies (depending on the quality of the previous transformation) of transformed *E. coli* BL21 (DE3) pLysS cells were removed and used to inoculate 5ml LB broth cultures. This was repeated until 7 independent cultures had been prepared. These were incubated in an orbital shaker overnight at 37^{o}C and 180rpm. 6 cultures of 500ml autoclaved LB broth with added ampicillin (to a final concentration of 50µg/ml) were preheated to 37^{o}C. The 500ml cultures were inoculated with 5ml of the overnight cultures, and grown to an OD_{600nm} of 0.6 at 37^{o}C and 180rpm. Once the OD of 0.6 was achieved, Isopropyl β-D-1-thianogalactopyranoside (IPTG, Melford) was added to each 500ml culture (to a final

concentration of 1mM) to induce expression. The cultures were left to express at 30^{o}C in the orbital shaker at 180rpm for 5 hours.

After expression, the 500ml cultures were centrifuged in a Beckman-Coulter Avanti centrifuge (using 500ml Beckman tubes in a JLA-10.500 rotor) at 15000g for 20 minutes, at a temperature of 4^{o}C. the supernatant was discarded, and the pellet was resuspended in 30ml of resuspension buffer (20mM TRIS pH 8.0, 150mM NaCl), and stored at -20^{o}C until use.

2.2.2.3. Cell lysis and Preparation of cell samples for purification

Resuspended cell samples were defrosted and sonicated for 6 minutes (5 seconds on, 5 seconds off, for a total of 12 minutes per sample) at 30mA. Sonication was carried out using a Fisher Scientific FB705 sonic dismembrator with a 3/16 inch probe. After sonication, the cell extracts were decanted into 50ml Beckman centrifuge tubes and pelleted using the Beckman-Coulter Avanti centrifuge (using the JA-25.50 rotor) at 30000g for 20 minutes, at a temperature of 4^{o}C.

The supernatant was decanted off and stored at 4^{o}C as the soluble fraction. The insoluble pellet was resuspended with 10ml of buffer (20mM Tris pH 8.0, 150mM NaCl, 20mM n-Dodecyl-β-D-Maltopyranoside (DDM)). This suspension was centrifuged using the same centrifuging conditions as the step before, to produce another supernatant and pellet. The supernatant was removed and stored at 4^{o}C as the DDM-containing fraction . The storage times for the soluble and DDM-containing fractions did not exceed 3 days, in order to limit ecoPBP1b degradation.

2.2.2.4. ecoPBP1b Purification

The DDM-containing fraction was loaded onto a 5ml HisTrap nickel column (GE Healthcare) at 1ml/minute for 3 hours by an AKTA Prime Plus (GE Healthcare), and was recirculated throughout. Whilst being loaded, the DDM-containing fraction was kept on ice.

Once loaded, the column was washed with 8 column volumes (40ml) of Buffer A (20mM Tris pH 8.0, 150mM NaCl, 1mM DDM), and the protein eluted with a 20 column volume (100ml) gradient of Buffer B (20mM Tris pH 8.0, 150mM NaCl, 1mM DMM, 1M imidazole). This gradient was a gradual increase of Buffer B concentration from 100%/0% of Buffer A/Buffer B respectively at the start through to 0%/100% of Buffer A/Buffer B at the 100ml mark. 10ml fractions were collected during the wash phase, and 5ml fractions were collected during the elution phase. Once purified, the fractions were collected and stored at 4^{o}C until used.

2.2.2.5. SDS-PAGE confirmation

In order to determine the fractions containing purified ecoPBP1Bb, samples of the fractions were run on a 12% SDS-PAGE gel. The samples were prepared as follows;

20µl of sample and 20µl of 2x SDS loading buffer (60mM Tris pH 6.8, 10% w/v of glycerol, 2% w/v SDS, 0.05% bromophenol blue, 5% w/v of β-mercaptoethanol) were added together. Samples were taken from the soluble fraction, DDM-containing fraction (prior to loading), flow-through and wash steps, as well as from the fractions collected after elution. Only 4µl of the DDM-containing and soluble fractions were used, and they were made up to 20µl with 18MΩ autoclaved water before 2x SDS loading buffer was added. Once mixed, the samples were heated to 95^{o}C for 3-5 minutes before being loaded onto the 12% SDS-PAGE gel. The gel was composed of a 12% resolving gel (3ml resolving gel buffer (1.5M Tris pH 8.8, 0.4% SDS v/v), 4.8ml 30% w/w acrylamide/bis-acrylamide solution (Sigma-Aldrich, product code A3574), made up to 12ml with 18MΩ autoclaved water, and polymerised with 100µl of 10% ammonium persulfate (APS) and 16µl N,N,N',N'-Tetramethylethane-1,2-diamine (TEMED)), and a 4% stacking gel (1.3ml stacking gel buffer (0.5M Tris pH 6.8, 0.4% SDS v/v), 0.7ml 30% acrylamide/bis-acrylamide, made up to 5ml with 18MΩ autoclaved water, polymerised with 50µl APS and 16µl TEMED). The gel was run at 60mA for 80 minutes, submerged in 1x SDS running buffer (25mM Tris, 192mM glycine, 0.1% SDS). A Precision Plus Protein Standard (Biorad) was also run alongside the fraction samples.

Once run, the gels were submerged in Coomassie Brilliant Blue stain (BioRad) (2g Coomassie Brilliant Blue Stain, 100ml acetic acid, 250ml propan-2-ol, 650ml 18mΩ autoclaved water), and microwaved for 30 seconds before being left to stand on a rotary table for 5 minutes. Once stained, the Coomassie was removed, and the gel underwent 3 changes of 1x destain (5% v/v propan-2-ol, 7% v/v acetic acid, 88% 18MΩ water).

Each change consisted of the gel being submerged in 1x destain, and left to stand for 15 minutes, before the destain was poured off. Gels were stored in 1x destain until imaged as required.

2.2.2.6. Concentration and Thrombin Cleavage

Fractions containing purified ecoPBP1b were concentrated down to a final volume of 5ml, using a Vivaspin 20 column (selective to 50kDa) (Sartorius). This was carried out using an Eppendorf 5810-R, with an S-4-104 square bucket rotor. RPM and time varied, from 1000-4000 rpm and 1-5 minutes respectively, depending on the volume required and rate of concentration. The 5ml protein solution was decanted into a 15ml falcon tube in preparation for thrombin cleavage. 3µl of Biotinylated thrombin (approximately 3 units, at 1unit/µl), alongside 200µl of 10x thrombin buffer (Novagen) were added, and incubated at room temperature for 16 hours.

Once cleaved, the 5ml of protein was manually loaded onto a 1ml HisTrap FF column (GE healthcare) to remove the cleaved His-tags, and the flowthrough collected in 1ml fractions. The column was then washed with 3ml of Buffer A, and eluted with 5ml of Buffer B, all collected in 1ml fractions. Using the previous method of SDS-PAGE, samples of the fractions were run on a 12% SDS-PAGE gel, in order to confirm the completion of the thrombin cleavage.

2.2.2.7. Buffer Exchange

Once the fractions containing purified cleaved ecoPBP1b were identified, they were collated together and concentrated using a Vivaspin 2 column (selective to 10kDa) to a volume of 1ml. 1ml of 4.5mM N-decyl-β-D-maltopyranoside (in 20mM Tris pH 8.0, 150mM NaCl) was added, the combination was subsequently concentrated to 1ml.

This was repeated (with a final total of 2ml 4.5mM N-decyl-β-D-maltopyranoside being used), before the same process was carried out with 0.28mM N,N-Dimethyldodecylamine N-oxide (LDAO) (in 20mM Tris pH 8.0, 150mM NaCl), with a final volume of 2ml of LDAO in resuspension buffer being used. These detergents and concentrations were identified as being able to maintain the stability of ecoPBP1b in aqueous solutions for crystallisation purposes in previous works of research (Sung *et al.,* 2009).

2.2.2.8. Crystallisation

After buffer exchange had been carried out, the protein solution was concentrated to 10mg/ml, quantified using a Nanodrop 2000 (Thermo Scientific), using the flowthrough from the concentrator to blank. The Nanodrop 2000 was set to measure absorbance at 260/280nm, using the extinction coefficient of ecoPBP1b (104.865 M^{-1} cm^{-1}) as well as the molecular weight (83.16kDa).

Once concentrated to 10mg/ml, ampicillin (50mg/ml, in a ratio of 1µl to 20µl of concentrated protein sample) and moenomycin conjugates (50mg/ml, in a ratio of 1µl to 20µl of concentrated protein sample) were added. 24 well plates with 1ml well volumes (Molecular Solutions) were used to set up the conditions for crystal growth. The crystallisation solution consisted of 1.22M sodium formate, as found by previous studies. Siliconised cover slips were polished using a silk cloth to remove dust and surface contaminants. 1µl of protein/ligand mixture was added to a siliconized cover slip, and 1µl of mother liquor (1.22M sodium formate) was mixed in. the slips were sealed into the tray wells using vacuum grease

(Dow Corning), and incubated at 16^{o}C for up to 2 weeks. Trays were checked after 5 days for signs of crystal growth, and trays with no growth at 2 weeks were removed. Trays with crystal growth were monitored until crystals were of a sufficient size.

Once fully grown, crystals were collected with loops and stored in a cryo-preservative solution (3M sodium formate) in liquid nitrogen. Chosen crystals were sent for X-ray diffraction data collection at the Diamond Light Source Synchrotron (Oxford).

2.2.2.9. Inducing Nucleation

It was noted in early trials that the numbers of suitably sized crystals were lower than expected. It was hypothesised that there may have been issues with nucleation occurring with ecoPBP1b, as a contaminated well containing a silk hair had grown crystals along the length of the hair (figure 9).

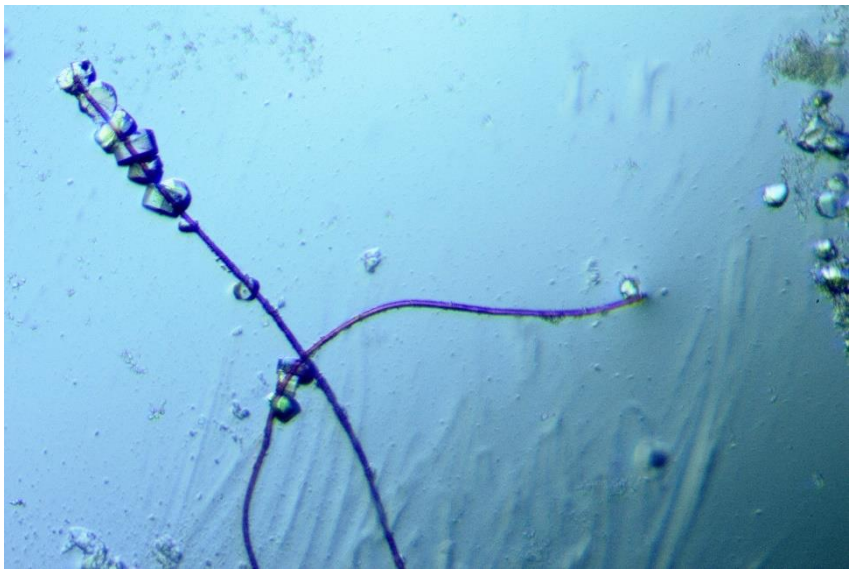

Figure 9. ecoPBP1b/AI168 crystals grown on a strand of silk in 1.22M sodium formate.

In order to improve the number of suitable crystals grown, a preliminary trial applying a variety of hypothesised nucleation techniques was formalised, partially based off the works of Georgieva *et al.* (2007). A 24 well plate was set up with 1.22M sodium formate, and the hanging drop method used as previously described. 4 techniques were utilised; 6 wells were

seeded using fragments of previously grown ecoPBP1b crystals, using a hypodermic needle to transfer the fragments from one drop to the next.

Another 6 wells had a single horse hair (chosen for the keratin structure) dragged through the protein/motherliquer drops. A further 6 wells had small fragments of horse hair deposited in the protein/motherliquer drops. The last 6 wells of the 24 well plate had fragments of silk deposited, from the same source of the contaminant hair of the previous crystal tray. Once the wells were sealed, the tray was left to incubate as previously described.

2.2.2.10. Diffraction Data Processing

X-ray data were processed automatically at the Diamond Synchrotron using xia 2. All Programs used to refine and produce a data set were found in the CCP4i Program suite. Xray Diffraction Data was used in conjunction with native ecoPBP1b dataset 3VMA and MolRep to solve the structure. An electron density map was calculated using RefMac, and automatic structural refinement was carried out using alternate cycles of RefMac and WinCoot. This model was then exported into PyMol for analysis and figure production. Distances between relevant residues and structures were measured in WinCoot.

2.3. Results

2.3.1. Protein Expression

The UV chromatograph data collected from the AKTA Prime Plus shows an increase in absorbance during the elution of the Histrap column with imidazole, as indicated by the chromatograph in figure 10. The SDS-PAGE gels (once stained) show good expression of ecoPBP1b (Figure 11), backing up the UV data. What is noted that the addition of DDM used in the resuspension of the initial insoluble pellet allows ecoPBP1b to be solubilised in resuspension buffer, as shown by the increase in the gel at 83kDa in the DDM-containing fraction (and lack thereof in the soluble fraction). The binding on the column is demonstrated by the visible decrease shown between the DDM-containing fraction (collected prior to column loading) and the flowthrough collected after repeated cycles. Later fractions collected from the addition of imidazole across a gradient shows large quantities of ecoPBP1b being expressed and successfully purified.

2.3.2. Thrombin Cleavage

Figure 12 shows good success in the thrombin cleavage. Large amounts of ecoPBP1b (shown at the 83kDa mark) are no longer binding to a nickel ion column, indicating that the histags have been successfully removed by the thrombin enzyme.

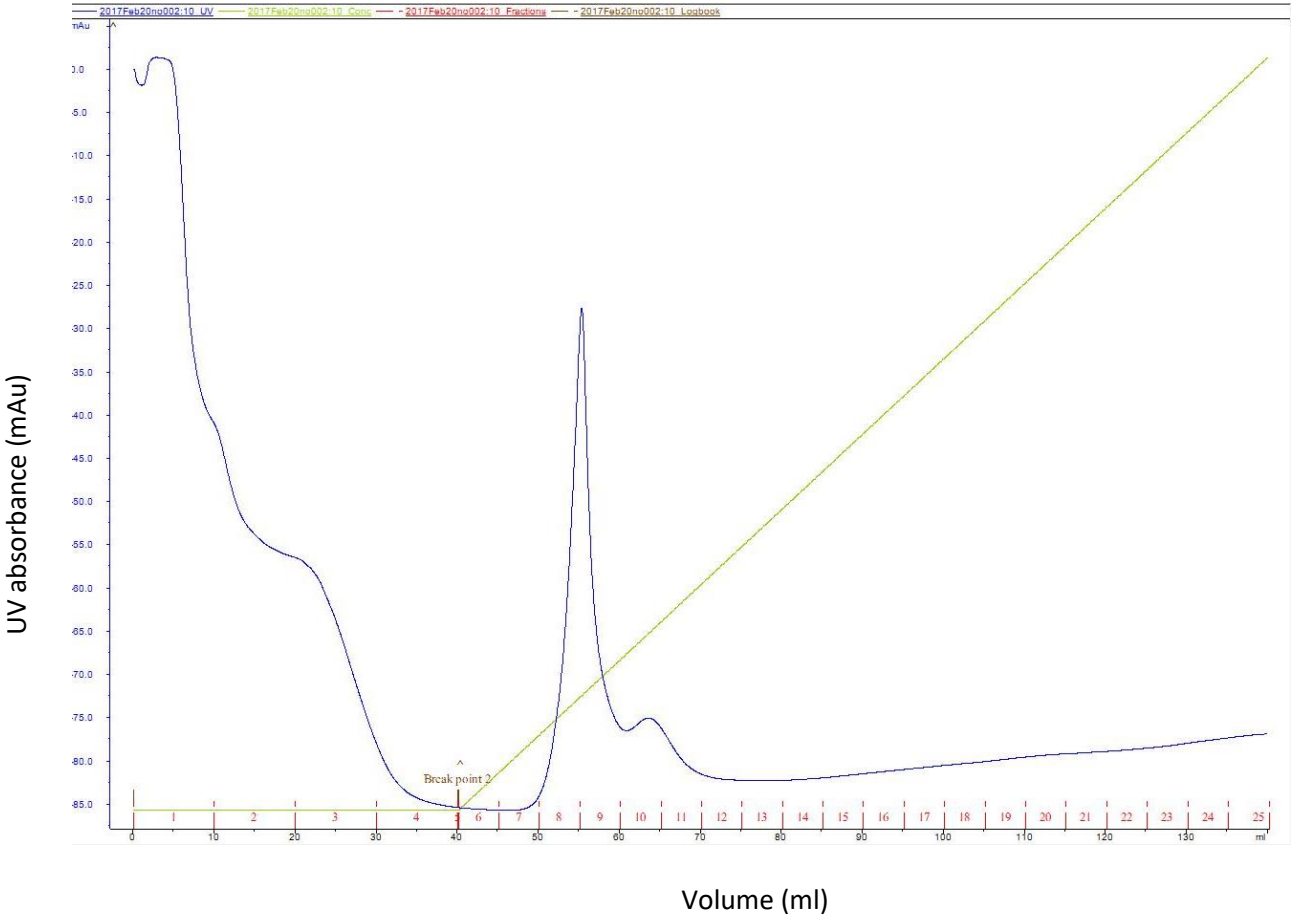

Figure 10. UV chromatograph trace showing the UV absorbance during the initial purification step using the AKTA Prime Plus. The bottom axis also shows the volume and number of fractions collected. The green line indicated the concentration of Buffer B (elution buffer) to Buffer A, which started to increase from 40ml onwards. Fraction 1 was used to represent the wash phase. Fractions 8 onwards were used for subsequent testing, due to the location of the peak. It should be noted that the peak doesn't directly correspond to the location and amount of the target protein, due to the weak binding of other proteins, and due to the UV absorbance of DDM.

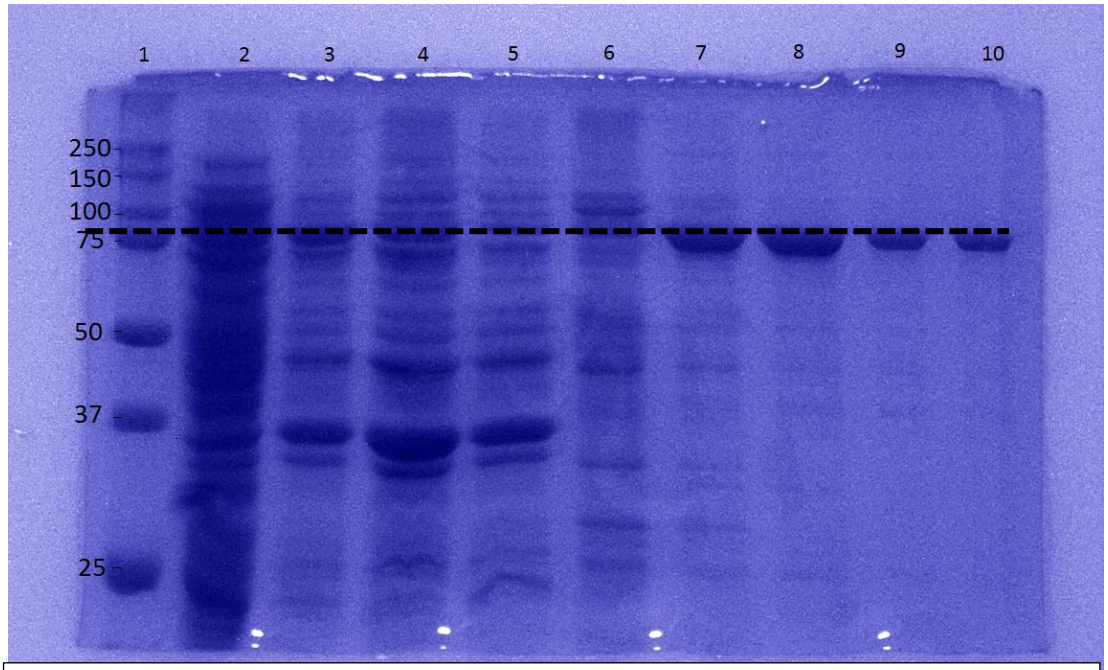

Figure 11. SDS-PAGE stained gel. Well 1; Precision Plus Dual Marker Protein Standard, Well 2; Soluble fraction, Well 3; DDM-containing Fraction (resuspended with 20mM DDM), Well 4; Flowthrough, Well 5; 1st Wash fraction collected, Wells 6-10; Fractions collected during elution, from the peak indicated on the UV spectra graph onwards. ecoPBP1b has a molecular weight of 83.19kDa, as indicated by the dotted line.

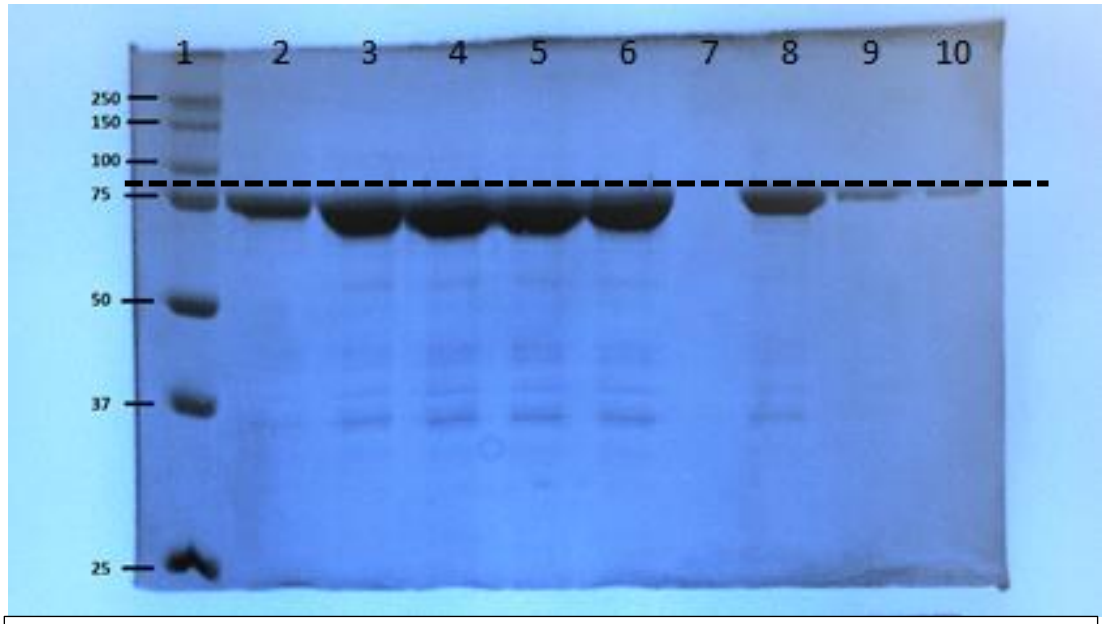

Figure 12. SDS-PAGE gel post thrombin cleavage. Gel 1: Well 1; Protein marker ladder, Wells 2-6; Flowthrough fractions of thrombin cleaved solution through nickel column, Wells 8-10; Wash fractions. Gel 2: Well 1, 3-6; fractions collected from column elution with 1M imidazole. The dotted line indicates the estimated size of ecoPBP1b (83.19kDa)

2.3.3. Crystal growth

Whilst successful in growing crystals, the number of crystals grown in early attempts was lower than expected, and many were appearing warped and twinned (Figure 13). It was hypothesised that there could have been nucleation issues.

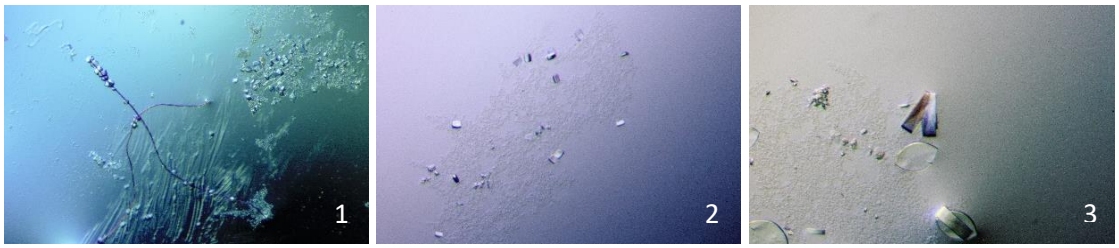

Figure 13. A selection of crystals produced in early ecpPBP1b/AI167p and 168p complex attempts. It should be noted that these are the more successful attempts at growing such crystals, many early trays had no clear growth. Image 1 shows crystal growth along a contaminating thread, which was an early inspiration in the seeding trials. In particular, image 3 shows 'twinning' of the crystals, which wasn't observed in later trials.

2.3.4. Seeding/nucleation Trials

Preliminary seeding trials were carried out, as previously described in the methods. The seeding method produced large quantities of crystals, at the cost of overcrowding in a number of the wells (figure 14).

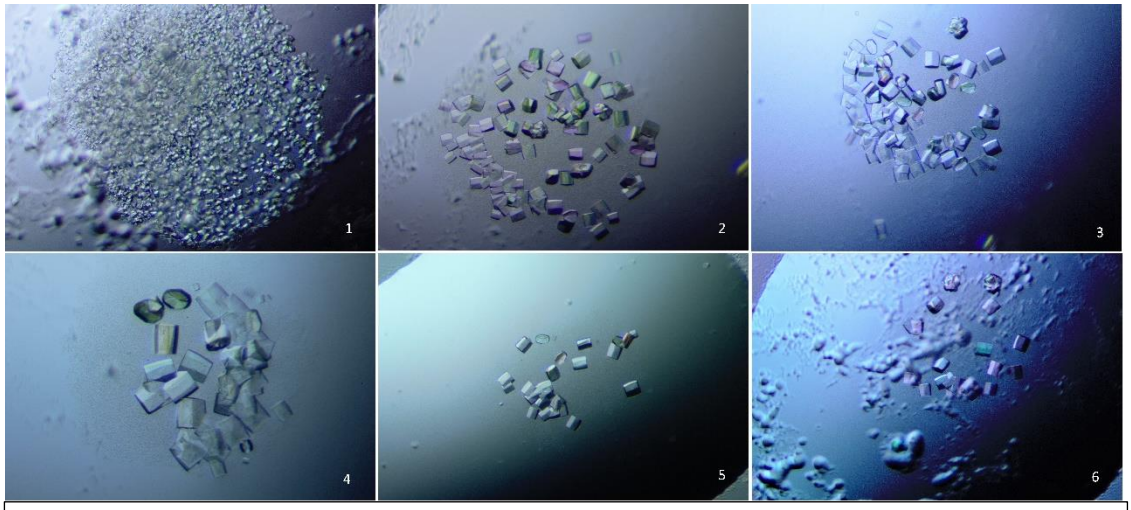

Figure 14. ecoPBP1b/AI167p complex crystals produced with the addition of seeding. The images are numbered in the other that they were seeded (Image 1 being the first well after crystal fragments were collected by the needletip, through to image 6 being the last seeded well. Crystals were taken from wells 3-6 for imaging.

The wells which had the strand of horsehair dragged through it, whilst showing some crystal growth, were not as increased as the seeding techniques, shown in Figure 15. Of the wells that had horse hair fragments in, only 2 showed any crystal growth, and of the crystals grown, none were of collectable quality (Figure 16). The wells containing strands of silk showed low to no growth during incubation, in contradiction of the initial expectations.

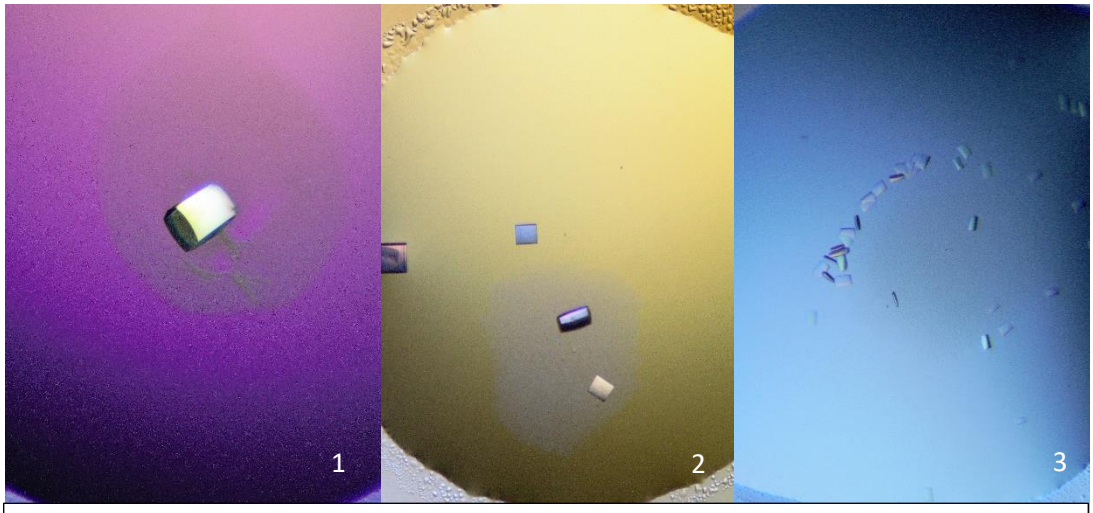

Figure 15. Crystals grown with horse hair dragged through the hanging drop. Larger crystals were observed in these trials, but the numbers of collectable crystals were not profoundly increased as compared to the original trials.

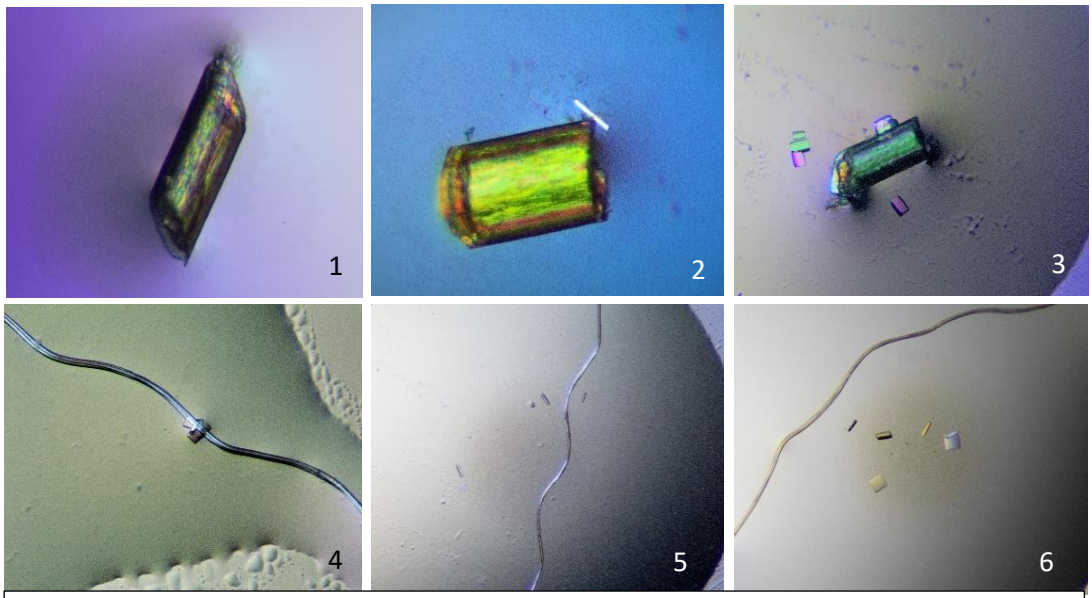

Figure 16. Images of the attempts to use fragments of horsehair and silk to induce nucleation and crystal growth. Image 1 shows precipitation but no growth around the hair fragment, images 2 and 3 show slight growth, but none of a suitable size. Image 4 shows a small amount of growth on the silk strand itself, but 5 and 6 show standard growth, irregardless of the presence of the silk strand.

Due to the increased crystal growth using seeding as a method to induce nucleation, subsequent crystal trays set up with ecoPBP1b/moenomycin conjugates also utilised seeding. This appeared to increased the output of crystals.

2.3.5. X-ray Diffraction and Structural Solving

Due to inherent poor diffraction, the crystals were screened at the Diamond Light Source Synchrotron. Those of the highest resolution were collected; 2 crystals grown in the presence of ligand AI168-p and one in the presence of ligand AI167-p. Of the 3 data sets, the one containing AI167-p had the best resultant structure. ecoPBP1b in complex with AI167p and ampicillin has been built, showing the binding of ampicillin (used to stabilise the ecoPBP1b structure) and the peptide subunits of MoeA (figure 217). However, the alterations made to MoeA cannot be seen. This implies that the alterations made to produce AI167-p and AI168-p do not alter the binding of these compounds to the transglycosylase domain of ecoPBP1b. The other collected structures (both containing AI168-p) showed no obvious differences in the binding of MoeA conjugates, and can be found in the appendices.

Resolution (Å)	2.72
Space Group	P: 2_1, 2_1, 2
Unit Cell dimension (Å)	63.410, 298.370, 62.480
Measured Reflections	21916
Unique Reflections	20624
Overall Completeness (%)	99.0837
Average Fourier Shell Correlation	0.825
Average Free Fourier Shell Correlation	0.8022
R_{factor}	0.2609
R_{free}	0.3233

Table 3. Diffraction Data and structural solving data for the ecoPBP1b crystallised in the presence of AI167-p and ampicillin.

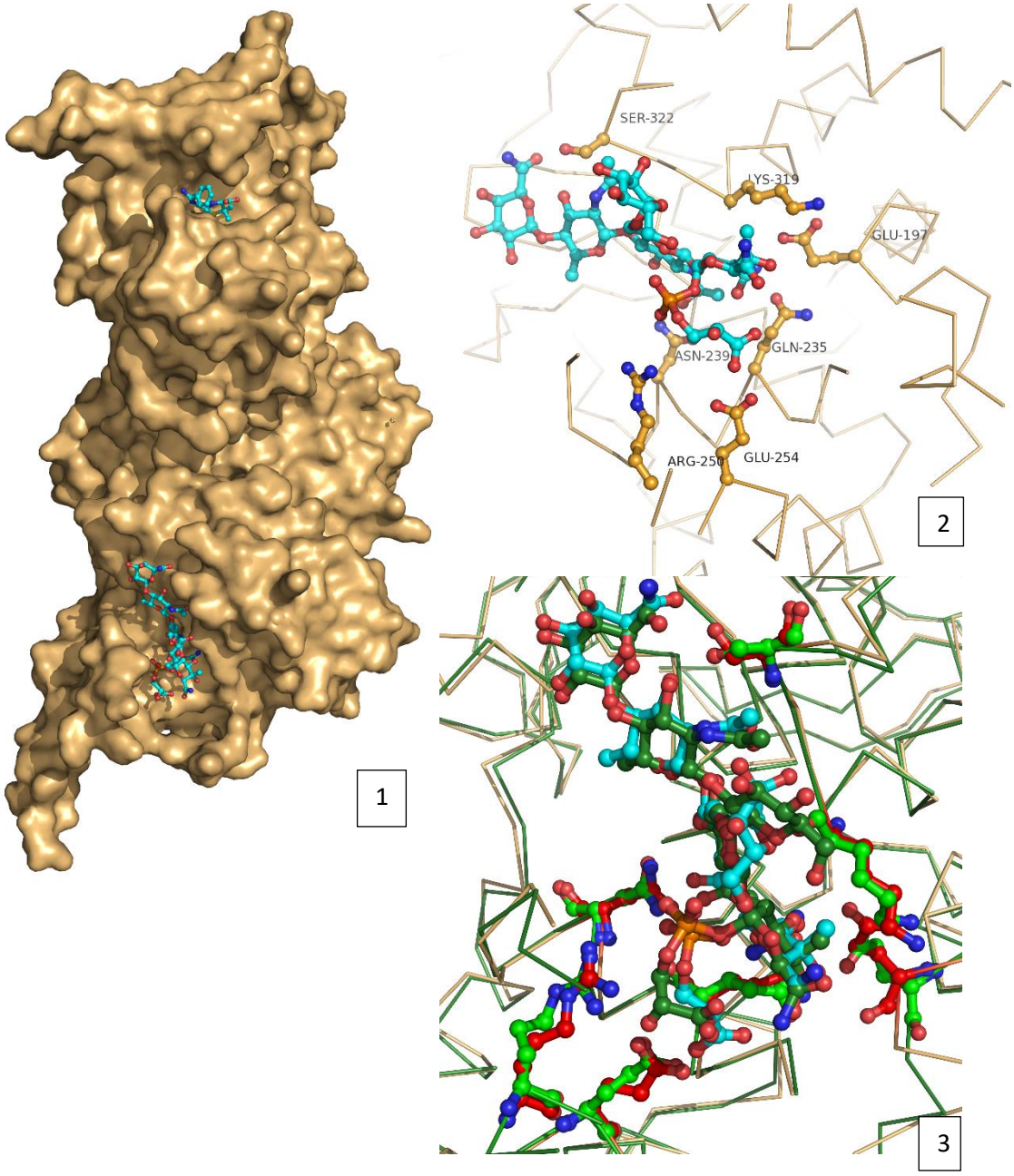

Figure 17. 3D models of ecoPBP1b with AI167p and ampicillin bound. 1; Full ecoPBP1b model, with ligands highlighted in blue. The top of the model shows ampicillin bound to the transpeptidase domain, and AI167p can be located at the bottom. 2; Close-up of the transglycosylase active site, with active residues identified in Sung *et al*. (2009) as involved in substrate binding labelled, and coloured in cream. 3; Overlay of the transglycosylase active site of ecoPBP1b (residues in red, AI167-p in blue) and 5HL9 (residues in light green, MoeA in dark green).

2.4. Discussion

2.4.1. 3D Structural Developments

The results show a structure of ecoPBP1b has been produced with clearly identifiable ligands bound. This structure allows for the visual identification of 3 of the 4 domains that ecoPBP1b is known to have; the transmembrane domain protruding from the base, the transglycosylase domain, as shown with the MoeA-based structure bound, and the transpeptidase domain (as indicated with ampicillin bound). The one domain not easily identifiable (UB2H) is still most likely there, as it is required for the stability of ecoPBP1b.

2.4.2. Active Site Interactions

What was highlighted in the results section was the portion of MoeA analogues that could be identified. The section of AI167 that could be characterised is the previously referred-to pharmacophore, with the lipid tail and alterations made disordered. The lipid tail is likely to be unbound, due to its role in binding to transglycosylases (i.e. acting as an identifier to allow access to the TG active site). As such, it may be disordered, unbinding once the MoeA has integrated into the TG active site. Being disordered means that it diffracts poorly or not at all, which implies that it has not bound at all, whilst the pharmacologically important sections (which have remained unaltered) are still able to definitively bind. This was confirmed with distances between the active site residues being measured, with 5 major residues (Glu197, Gln235, Asn239, Glu254, and Ser322) showing distances between 2.52-2.81Å, with two residues (Arg250 and Lys319) showing a binding above 3.20Å, measuring at 3.25Å. Matching the findings of previous studies in to the TG domain of PBP1b, we found that the moenomycin pharmacophore is stabilised by hydrogen bonding with at least 5 residues (those measuring in at <2.90Å), with weaker Van der Waals interactions from Arg250 and Lys319 helping to further facilitate binding whilst allowing a certain degree of movement (necessary for the polymerisation nature of the TG domain).

What is of more significance is the lack of presence of any peptide alterations made to the A ring of MoeA. The changes made to the analogues being tested are primarily based around improving bioavailability as well as improving the ability of MoeA to inhibit Gram negative species. The primary modification made to MoeA were linked peptides designed to facilitate the entry of the MoeA molecule into the periplasm of Gram-negatives. This means that the A- ring is a suitable region of MoeA to link changes to, with minimal disruption seen when bound to the TG domain. It is positive to notice that any changes made to alter the conformation of MoeA in solution (i.e. folding the lipid tail into the centre of the peptide structure, preventing binding to unintentional targets) haven't prevented MoeA from binding in its original manner.

2.4.3. Comparison to previous ecoPBP1b structures

In addition, a comparison of the ecoPBP1b structure produced as part of this project was compared to 5HL9, characterised recently by King *et al.* (2016) 5HL9 was produced in complex with native moenomycin, as well as ampicillin in the transpeptidase domain. When the structures were overlaid in PyMol, clear similarities could be seen. Other than small conformational differences (which are to be expected, due to the differences in moenomycin analogues), our model corresponds well with this previous data set, including the amino acid residues located in and around the active site.

2.4.4. Crystallisation of ecoPBP1b: Has it been improved?

An identifiable concern early in the project was the sparse numbers of crystals produced in early trials. Having run multiple expressions, the distinct lack of protein crystals was a worry. It had been suggested that there could be issues with the nucleation of the crystals. Many studies refer to two distinct types of nucleation; homogenous nucleation (where the crystals form spontaneously providing the conditions are correct) and heterogenous nucleation (where the crystals form on the surface of solids or particles present in the motherliquer).

The methods used in early trials relied on homogenous nucleation to occur, using the handing drop diffusion method, based on conditions found in previous research projects. In one particular trial (as mentioned in the methods), protein crystals grew along a single strand of silk. This suggested that whilst the conditions were suitable for crystal growth, there could be higher yields if ecoPBP1b complex crystals could be grown using heterogenous nucleation methods.

Building off numerous studies looking into inducing nucleation (most notably Georgieva *et al.*, 2007), three different nucleators were used; fragments of previous ecoPBP1b crystals, horse hair (using 2 distinct methods), and threads of silk from the same source as the contaminant.

One thing which was particularly surprising about these trials was not the success of seeding; seeding using fragments of crystals is a common method of improving the yield of protein crystals, as it provides a partially constructed scaffold for the asymmetric protein units to build upon. Instead, it was the lack of visible crystal growth in any well containing silk thread. This was in direct contrast to the previous attempts to grow crystals, where the silk thread was one of only a few wells to produce significant crystal growth (albeit crystals bound to the thread themselves which, due to the fragility of the crystals, were not collected for data processing). It could potentially be that the thread contaminant was mis-identified, so the addition of other types of fabric thread would produce a misleading result.

The moderate success of the horse hair trials appears to back up the findings of previous papers, such as Georgieva *et al.*, (2007), but not to the extent that was expected. The hypothesised cause of the nucleation induction in this method are microfragments of keratin which are deposited when the hair is dragged through the mother liquor. These microfragments are hypothesised to cause protein in solution to deposit on the surface, beginning the steps of crystal formation. Whilst there was crystal growth above the levels

seen in prior trials, it was not high enough to warrant further study (certainly, not compared to the fragment seeding trials).

One interesting hypothesis that occurred during these trials is the possibility that homogenous nucleation is not as random and spontaneous as expected. The majority of methods relying on homogenous crystal formation rely on the protein achieving a so-called 'supersaturated' concentration in mother liquor. There remains the potential that when this state is achieved, what is occurring is that minute quantities of the protein are precipitating out and aggregating, but not to the extent of triggering mass precipitation. Instead, the aggregation could well form a seed from which a crystal could grow. It is not yet known how to test this hypothesis, but it is certainly an interesting possibility to consider, as it could improve crystal yields in future experiments.

3. Beta Lactams

3.1. Introduction

The other class of antibiotics studied in the process of this research are the beta-lactams. Beta-lactam-based antimicrobials have been used in clinical settings for decades, and their mode of action has been known for years, binding to a group of enzymes involved in the transpeptidase stage of peptidoglycan synthesis. These proteins are classified as Penicillin-binding proteins (PBPs), the majority of which were identified following studies into the exact activity of penicillin. The majority of research into beta-lactams and their mode of action was carried out in the 1970s and 1980s (Spratt, 1975; Strominger et al., 1971; Waxman and Strominger, 1983).

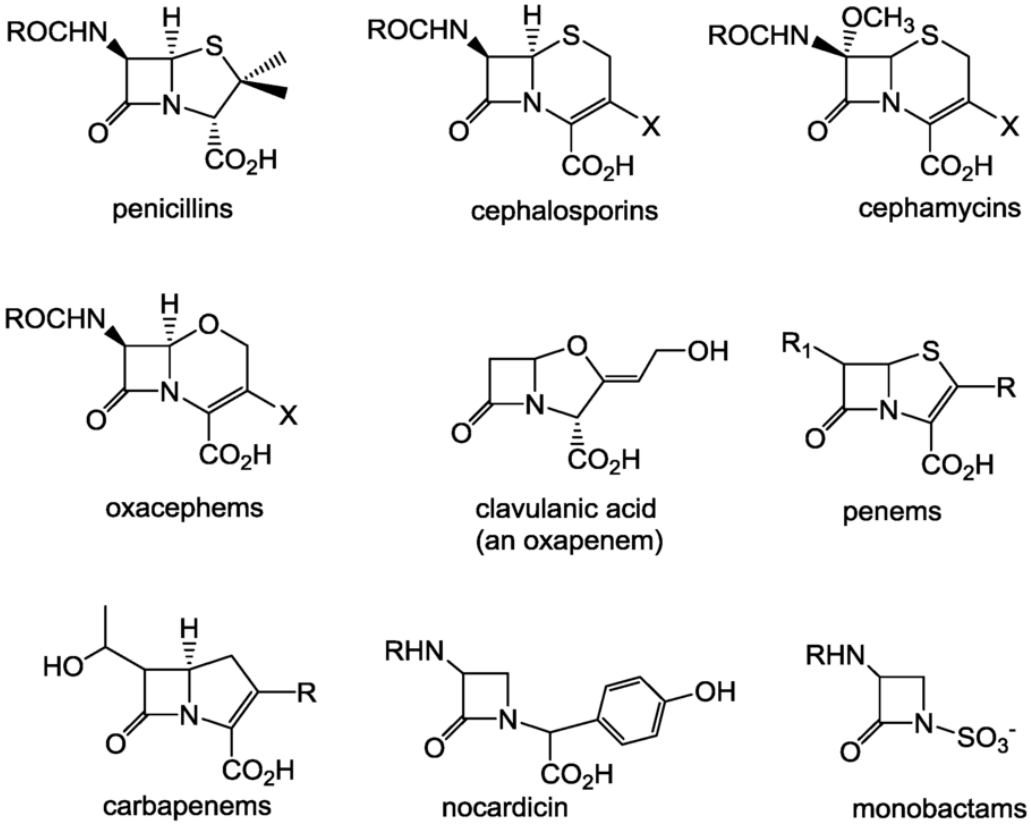

Figure 18. Clinically relevant beta-lactam antibiotics. The majority are bicyclic (with 2 rings forming the pharmacophore), with others being monocyclic, such as nocardicin and the monobactams. Reproduced from Konaklieva (2014).

3.1.1. Beta-Lactam Characteristics and Mode of Action

Beta-lactams are defined by the inclusion of the beta-lactam ring, show in figure X. This beta-lactam ring, produced by fungi to mimic the D-ala-D-ala termini found on peptidoglycan recognised by transpeptidases (Figure 19). Mimicking this structure allows access to the active site of such PBPs, where the antibiotic can bind to a catalytic serine residue. This serine opens up the beta-lactam ring, producing an acyl-enzyme complex in the same manner that it would normally bind to peptidoglycan (Park and Rafii, 2017). This beta-lactam version acyl-enzyme complex disrupts the PBP's ability to synthesise the peptide bonds between nascent chains, as it prevents the second D-ala-D-ala (required for the crosslinking process) from binding (Kotra and Mobashery, 1998). Once inhibiting PBPs and being incorporated into the peptidoglycan structure, beta-lactams tend to cause cell lysis. This lysis is caused by the combination of naturally occurring cellular expansion combined with the weakened peptidoglycan, and results in the killing of the bacterial cell.

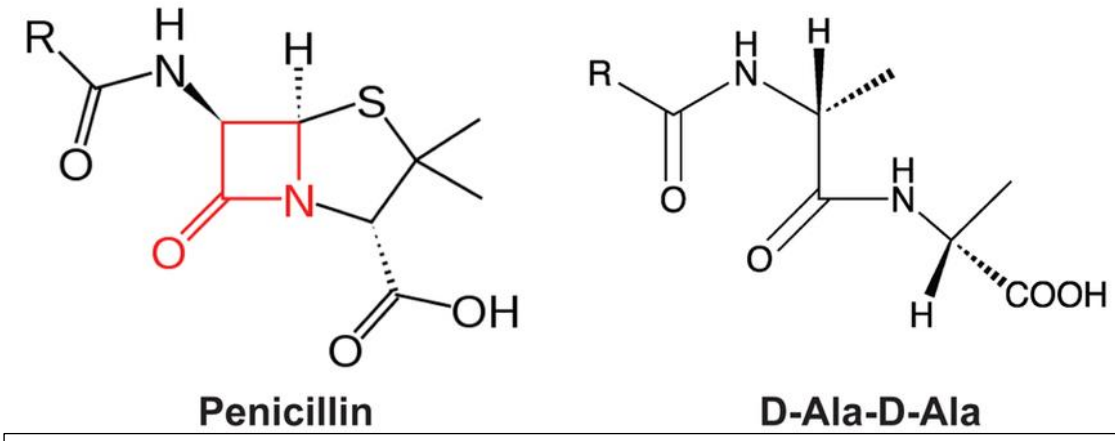

Figure 19. Demonstration of the similarities between penicillin and the D-Ala-D-Ala termini. The beta-lactam ring has been highlighted in red. This image was reproduced from Zeng and Lin, (2013)

3.1.2. Development of Resistance

Resistance to these compounds comes from 3 predominant mechanisms; production of efflux pumps, alterations of PBPs to lower beta-lactam binding affinity, and the production of beta-lactamases (Konaklieva, 2014). Efflux pumps have been seen to develop in beta-lactam resistance, but have limited effects, due to the location of PBPs close to the outside

of bacterial cells (Pan et al., 2016). Altered PBPs additionally present an issue, and can be harder to treat, as beta-lactams will be unable to bind to the modified PBPs. There must be limits to such modification, as PBPs still require the ability to bind the D-Ala-D-Ala termini to function properly, so such modifications tend to revolve around making the active site harder to access. Whilst the first two noted (efflux pumps, altered PBPS) have an appreciative effect on organism survival, the most important clinically are beta-lactamases. These specifically target the beta-lactam ring, inactivating this structure through hydrolysis. The exact number of beta-lactamases currently identified varies, with recent estimates of upwards of 2000 (Bonomo, 2016).

3.1.3. Beta-Lactamases

Beta-lactamases can be subdivided using the Ambler system into 4 categories based on conserved amino acid motifs; A, B, C, D. These can be broadly split into 2 major groups: enzymes which utilise a catalytic serine to form an acyl complex with the beta-lactam ring (categories A, C, D), and those which require at least one active-site zinc molecule (category B, also known as the Metallo-B-lactamases) (Bush and Jacoby, 2009).

A significant concern with beta-lactamase-mediated resistance is the extended spectrum nature of such enzymes, not only for antibiotic classes but also for bacterial species. Many beta-lactamases have effects against multiple beta-lactam classes, due to the conserved pharmacophore of many of these antibiotics. In addition, many MDR plasmids (also known as MDR cassettes) contain beta-lactamase genes (such as the $bla_{\text{CTX-M-15}}$ gene commonly found in resistant *E. coli* strains) and are able to transfer horizontally (Wilke, Lovering and Strynadka, 2005). Horizontal gene transfer enables increased development of resistance (as compared to *de novo* resistance), as well as the possibility for species crossover (Ochman, Lawrence and Groisman., (2000); Sieber, Bromley and Dunning Hotopp, (2017)).

3.1.4. Overcoming Resistance to Beta-Lactams

The development of such resistance prompted an ‘arms race’ between scientists and bacterial species, producing antimicrobials to avoid beta-lactamase degradation (such as methicillin), or developing beta-lactamase inhibitors such as diazabicyclooctanes (Wright, 1999). Many of these inhibitors are beta-lactam mimics, which are able to target beta-lactamases directly, but many have little to no direct antimicrobial activity (Coleman, 2011).

The development of beta-lactams has been a cornerstone of antibiotic research since the aforementioned discovery of penicillin. Due to this, many of the new compounds are structurally similar to their predecessors. This restriction of diversity in the compounds being pushed for clinical usage increases the probability that resistance will develop to these new compounds. Being structurally similar means only minor mutations are required for the previously mentioned resistance mechanisms (Altered PBPs, transporter proteins, beta lactamases).

One solution to this problem is to develop beta-lactamase inhibitors to be used in conjunction with beta-lactam compounds. Such compounds with beta-lactam derived structures specifically designed to target these beta-lactamases, preventing the degredation of beta-lactams before they bind to PBPs. Due to their similarities to beta-lactam structures, it could be hypothesised that such inhibitors could potentially bind to PBPs with inhibitory effects.

3.1.5. Aims and Objectives

This chapter is based around whether different Beta-lactams bound to the penicillin binding region of the TP domain of ecoPBP1b cause differing active site interactions. The antimicrobial compounds used were all chosen due to size and structural variations away from the identified pharmacophore. In addition, a novel beta-lactamase inhibitor was

studied, looking to see whether the changes to the beta-lactam core would still allow it to bind to the transpeptidase domain.

Specifically, this chapter looked at one major question; Do differing beta-lactam compounds produce major changes in the active site interactions required for binding to the transpeptidase domain?

In order to answer this question, structural biological techniques were used in order to study the binding of beta-lactams to the penicillin binding region.

3.2. Alterations to the Methodology

The methods used in this chapter were almost identical to those used in the moenomycin chapter, with one major difference: the usage of different beta-lactams other than ampicillin used to bind to ecoPBP1b's TP domain. Native ecoPBP1b without ampicillin or MoeA was found to be unstable, so the addition of compounds from both families was essential. As crystals had been produced with AI167 and AI168, these compounds were used interchangeably to stabilise the TG domain, whilst different beta-lactam based compounds were used as ligands for the TP domain.

3.2.1. Crystallisation

Once purified ecoPBP1b had been concentrated to 10mg/ml in LDAO buffer, the moenomycin A conjugates were added (10mg/ml stock, in a ratio of 1µl to 20µl of protein sample), as well as the chosen beta-lactam (50mg/ml stock, in a ratio of 1µl to 20µl of protein sample). The beta-lactams used were; amoxicillin, ampicillin, cefotaxime, cephradine, oxacillin and penicillin G. They were selected due to their variances in structure, as well as availability (Figure 20). In addition, a novel beta-lactamase inhibitor was trialed, replacing the beta-lactams, and using the same stock concentrations and ratios. Trays were set up in accordance with the previous methodology, as were any further steps taken.

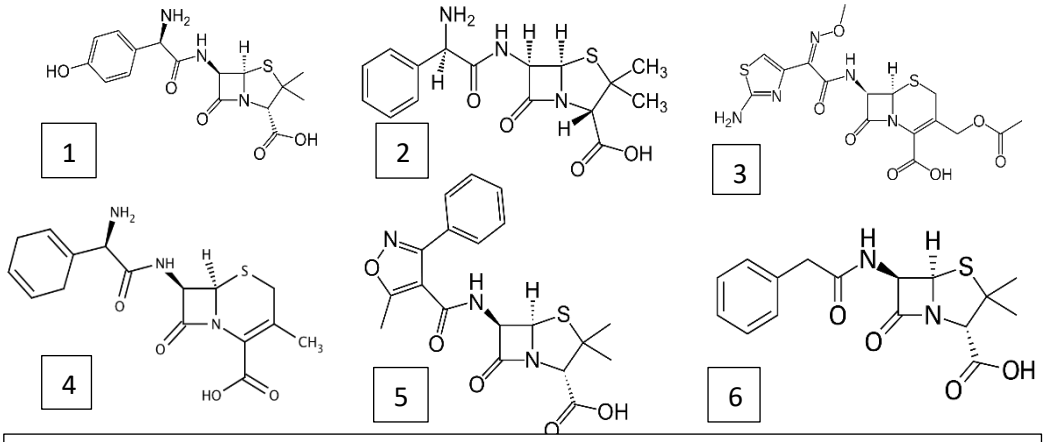

Figure 20. Structures of the beta-lactams used during this project. 1, Amoxicillin; 2, Ampicillin; 3, Cefotaxime; 4, Cephradine; 5, Oxacillin; 6, Penicillin G.

3.3. Results

As previously stated, the methodology used was identical to the one used in the moenomycin section. As such, this results section is to cover the results where the methodology started to vary; crystal production with a variety of ligands.

3.3.1. Crystal Production

The majority of the crystal trays set up with the beta-lactams failed to produce any crystals. However, crystals were formed in wells containing cefotaxime and cephradine. Crystals were

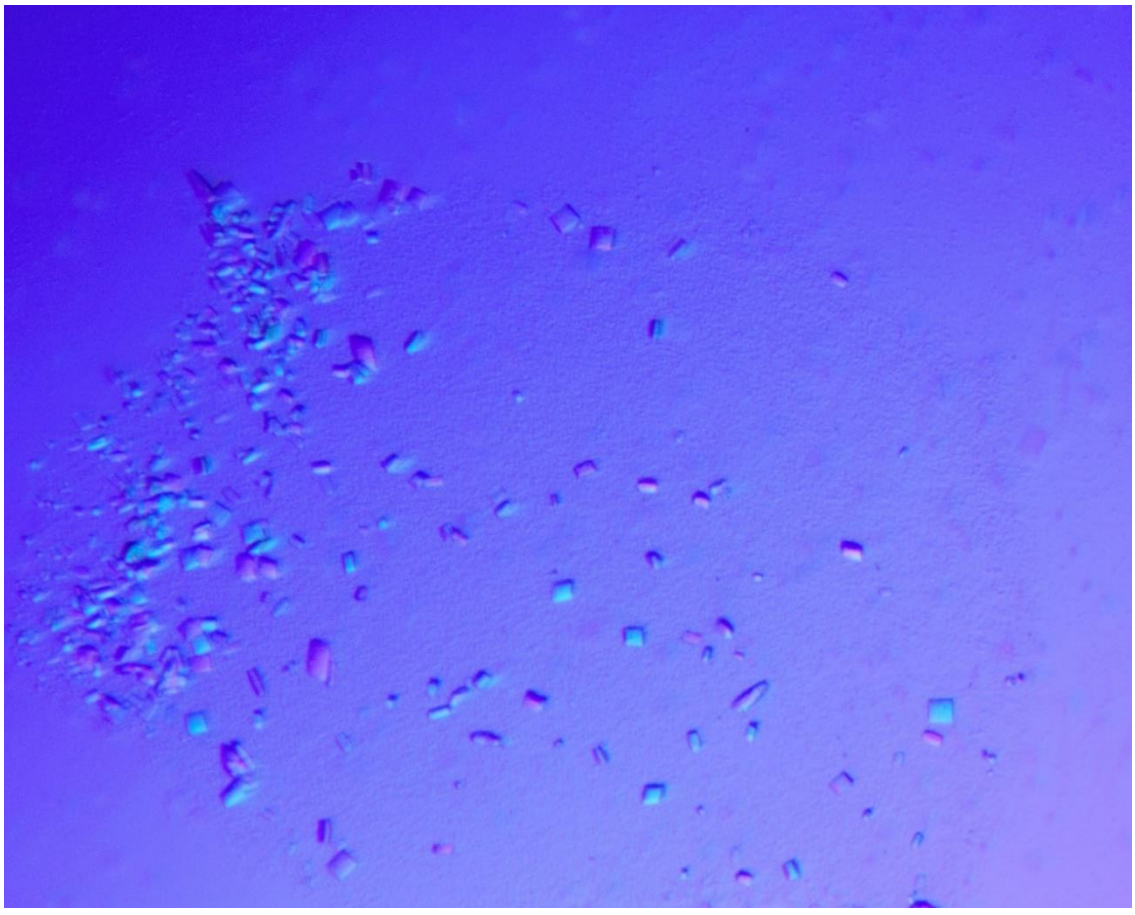

Figure 21. Crystals formed in the presence of ecoPBP1b/AI167p/cefotaxime. The lack of regularity should be noted in these crystal structures. Many of the crystals collected from this well were found to be salt crystals.

grown in the presence of the novel beta-lactamase CW-019, and further work is being carried out with these crystals.

3.3.2. Diffraction Data sets

Crystals suspected of containing cefotaxime and cephradine respectively were sent for data collection at the Diamond Light Source Synchotron. Whilst a data set was collected for a crystal potentially containing cefotaxime, due to the poor resolution of the diffraction data, it was decided not to attempt structural solving. The crystals suspected of containing cephradine produced diffraction data sets which appeared to be salt crystals as opposed to protein crystals. As such, this project produced a structure of ecoPBP1b in complex with one beta-lactam; ampicillin.

3.3.3. 3D modelling of the Penicillin Binding Region

Using the data collected as part of the moenomycin chapter, the penicillin binding region was identified, as well as some potential key amino acid residues. Using the data from King *et al.* (2016), the potential catalytic residues were identified, bond distances measured using WinCoot, and modelled in PyMol (figure 22).

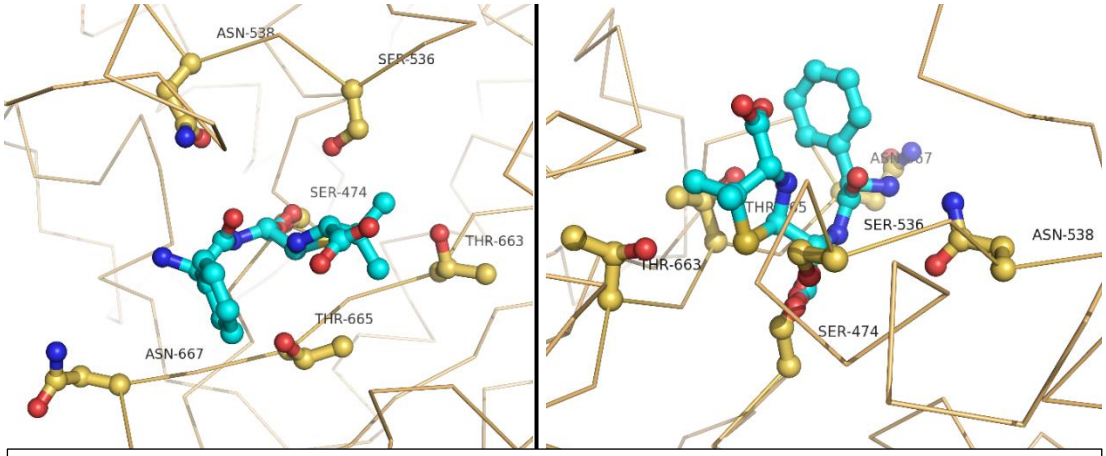

Figure 22. Ampicillin (Blue) bound to the transpeptidase binding region of ecoPBP1b. Labelled are the amino acids that King *et al* (2016) identified in their model of PBP1b as necessary. Ampicillin binds to a serine residue (Ser474) and is stabilised by 5 other residues. These 5 residues varied in distance from 3.05-3.59Å (with the exception of Asn677, which was further away, with a distance of 5.18-5.20Å)

3.4. Discussion

3.4.1. ecoPBP1b and Ampicillin

The model shows ampicillin covalently bound to Ser474 in the penicillin binding region, with overlapping residues. The calculated distances support the theory that the other residues (Ser536, Asn538, Thr663, Thr665 and Asn667) do not strongly interact with the beta-lactam, but rather fold around, enclosing the beta-lactam molecule into a 'cleft'. Whilst Asn667 is considered part of the active site, the distance between it and the ampicillin molecule is larger than 4.0Å, indicating that there is no direct interaction. It is possible that Asn667 plays an active role in peptidoglycan polymerisation, but is unable to bind to a beta-lactam compound.

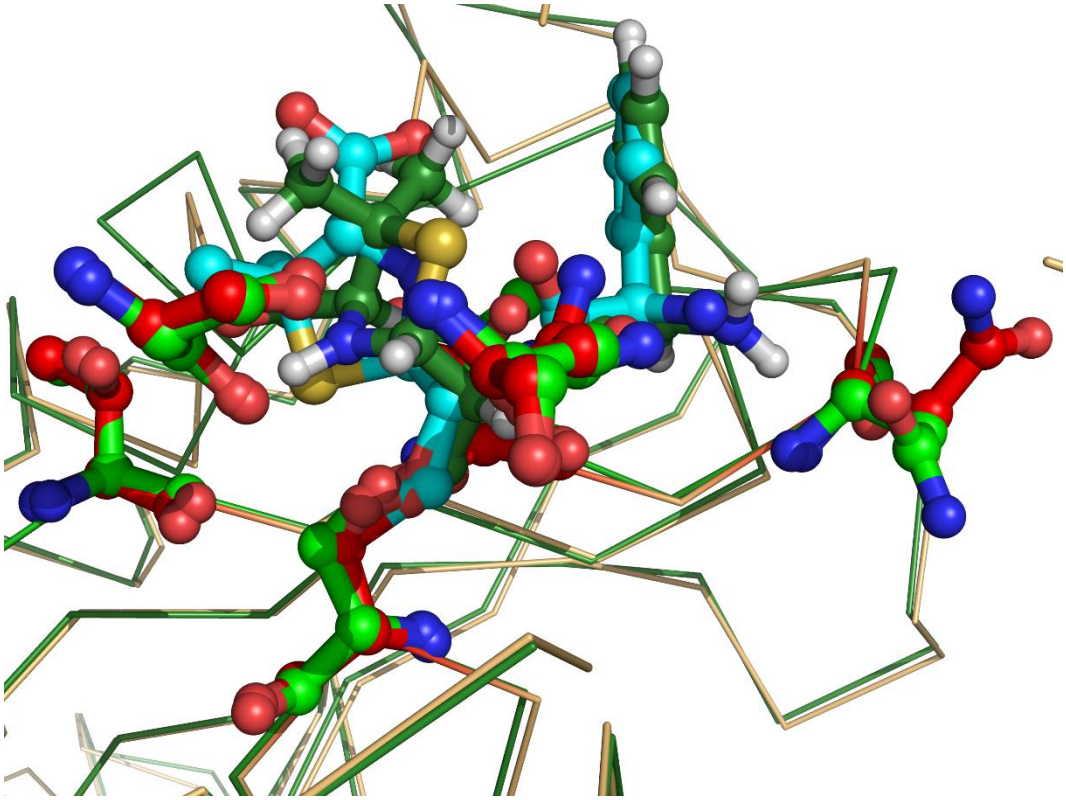

Figure 23. Overlaid structures of ecoPBP1B's transpeptidase domain (active residues in red, ampicillin in blue) and 5HL9 (residues in light green, ampicillin in dark green) from King *et al.* (2016). The overall matching is good, with clear similarities, and no missing residues. Noted is the residue to the far right (Asn667), which is rotated in comparison to the 5HL9 model.

Our model with ampicillin corresponds well with the data from King *et al,* (2016). Overlaying the structure of the transpeptidase region shows good overall pairing, as well as the specific residues in the active site (Figure 23). One residue that stands out is Asn667, which is configured differently to the 5HL9 model. Due to the distance between the residue and the ampicillin, its indirect nature (for this substrate) may allow for more direct binding with penicillin binding substrates containing different functional groups. Alternatively, it could be a redundant conserved residue required for the function of PBPs other than PBP1b, as noted by King *et al.* (2014) who saw involvement with the Asn residue for the PBP3 found in *P. aeruginosa*.

3.4.2. Beta-Lactam Crystal Production

It would be useful to understand the alterations caused to the conformation of ecoPBP1b with different beta-lactams bound. Whilst structures of ecoPBP1b in complex with ampicillin were produced, no other beta-lactam trials reached this stage. ecoPBP1b crystals are notoriously hard to collect data sets of, due to their poor diffraction and overall fragility. Many crystals have been grown containing a variety of compounds, but due to the poor diffraction, it was decided to only use crystals of a certain size for diffraction data collection.

This contrasts with the previous chapter, where ecoPBP1b crystals in complex with multiple ligands had been produced and successfully diffracted at a suitable resolution. This suggests that there is issue with the beta-lactams complexing with ecoPBP1b. However, the lower number of trials as compared to the previous chapter should be noted, as a larger proportion of project time was spent on producing ecoPBP1b/MoeA/Amp complex crystals with modified moenomycins. Had an equal amount of time been spent on the beta-lactams project, it is probable that sizeable crystals would be produced.

3.4.3. Potential Issues with EcoPBP1b as a model for Beta-lactams

Due to the structural nature of ecoPBP1b, with multiple domains (including one specifically designed to penetrate lipid-based membranes), it is not a simple protein to characterise. It requires the addition of multiple ligands to stabilise the structure (a moenomycin-based compound in the TG domain, as well as a beta-lactam in the TP domain). What has been demonstrated is the ability of the TG domain to accommodate ligands with varying structural alterations, providing the moenomycin pharmacophore is conserved. This has not yet been substantiated with regards to the TP beta-lactam binding site. However, it is unlikely that the beta-lactam compounds trialled are of sufficient structural difference to ampicillin as to cause major instability. The compounds tested had various side group differences, but all contained the conserved beta-lactam ring which comprises the beta-lactam pharmacophore. As the compounds used all are currently or have previously been approved in clinical settings (in addition to having well documented modes of action), it is unlikely that their attachment to the penicillin binding region of the TP domain would have caused significant instability. In addition, none of the trials showed significant precipitation early on in the crystallisation waiting period, something that had occurred in previous trials when ecoPBP1b wasn't sufficiently stable. What is more likely is that the addition of other beta-lactams has caused conformational shifts which require differing conditions for crystallisation to occur.

The idea that ecoPBP1b could be used as a model for beta-lactams has not been substantiated with this experiment, due to the lack of structures produced. However, significant amounts of the groundwork necessary to test this idea has been achieved, with a fully functioning methodology for producing ecoPBP1b produced from this project.

3.4.4. Advantages and Disadvantages of Monofunctional Models

It may also be worth using a monofunctional transpeptidase (preferentially a cytoplasmically accumulated recombinant transpeptidase, which doesn't have the issues related to

membrane-bound transpeptidases) to model the changes in conformation. Such enzymes may be simpler to express and characterise, and would still provide an understanding of the conformational changes caused by side chain variation in beta-lactams. However, the disadvantage of this is the inability to compare the quaternary structures of a bifunctional glycosyltransferase, and how it is affected by beta-lactams. Depending on whether future projects were based around further understanding ecoPBP1b (or homologues) or more based around developing novel beta-lactam based compounds, using a monofunctional transpeptidase may be recommended.

4. Overall Discussion

This project has been successful in a variety of ways. Firstly, the confirmation that the MoeA conjugates have had no visible alterations to the active site interactions, in conjunction with MICs from other research projects, is a good sign for further development of improved moenomycin antimicrobials. In addition, it verifies the use of ecoPBP1b as a model for novel moenomycin compounds, to test mode of action. In addition, this project has improved the methods to obtain useable quantities of ecoPBP1b for further research, especially for X-ray crystallography.

4.1. Improving the Yield of ecoPBP1b crystals

The utilisation of seeding to dramatically improve the yield of ecoPBP1b crystals can also be considered a success. Improving the numbers of suitable crystals increases productivity and efficiency, which is useful. It should be taken into account that the crystal seeding did not particularly improve the numbers of suitable crystals in the beta-lactam chapter, and there is always the risk of overseeding. Overseeding can lead to crowding of the trial wells, and prevent crystals from reaching optimum sizes. Whilst not particularly seen in the beta-lactam chapter, it was more obviously shown in the MoeA chapter, with 2 wells of 6 being overcrowded (leading to a need to seed down a gradient, to produce wells of optimal crystals).

4.2. ecoPBP1b as a Model for Antimicrobials

The overall failure to use ecoPBP1b as a model for multiple beta-lactams is an area that requires further work. The aim of this project was to produce a variety of structures with different beta-lactams bound to the penicillin binding region, in order to look for variations in conformation change, highlighting the effects that different residues have. There is the potential that the ecoPBP1b/beta-lactam complex may require altered crystallisation conditions, with the current conditions preventing proper crystal growth (resulting in the

significantly smaller crystals seen in the previous chapter). It is possible to use ecoPBP1b to model beta lactams, as demonstrated by King *et al.* (2016), who characterised ecoPBP1b with compounds such as acyl-ampicillin, cephalexin, CENTA, and aztreonam (as part of a wider study). This implies that such a model is achievable. It should be noted that the methods used by King *et al.* varied in their crystallisation stage, in which they utilised the sitting drop method in addition to differing crystallisation conditions (their mother liquor consisted of PEG 3350 (20% w/v), potassium/sodium tartate (0.2M), and Bis Tris (0.1M, pH 8.5), whereas the precipitant used in this project was sodium formate (1.22M). Furthermore, the concentration of ecoPBP1b used by King *et al.* was significantly higher than this project (20mg/ml as opposed to 12mg/ml).

Regarding the concentration, this may be due to the detergent required for the sodium formate precipitant (in this case, LDAO). Differing detergents may limit the concentration that can be achieved. During the process of concentrating after buffer exchange, it was noted that a concentration above 13mg/ml caused issues in the crystals produced (warped crystals, overprecipitation). It may be worth utilising the sitting drop method in the conditions described above in future trials.

Additionally, the use of detergents is well known to interfere with the detection and quantification of purified proteins (Simonian and Smith, 2006). Many detergents, when not bound to protein molecules, form micelles in solution, which often absorb light at a wavelength of 280nm. This can result in elevated readings, which may cause later issues (if the concentration of protein is lower than calculated, then it is less likely that a supersaturated solution will be formed during crystal growth trials.

In order to compete with simpler enzymatic models (the monofunctional TGs and TPs spring to mind), issues such as these need to be addressed. At this stage, ecoPBP1b can be used to

model novel MoeA analogues well, but improvements are required before it can be used as a model for beta-lactams (or other similar classes of antimicrobial).

4.3. Beta-lactamase Inhibitor Binding

The beta-lactam chapter also included trials to attempt binding of a novel beta-lactamase inhibitor to the transpeptidase domain, in order to determine if it would have the side effect of also acting as an antimicrobial agent. As noted in the results of the beta-lactam chapter, crystals have been grown in the presence of ecoPBP1b and CW-019, and research is still ongoing.

Beta-lactamase inhibitors tend not to have antimicrobial properties, due to their altered structure (many containing significant variations to the beta-lactam core). When the crystal trays were set up, ampicillin was replaced with the CW-019, as ampicillin would have displaced any CW-019 when competing for the binding site. If CW-019 does not bind to ecoPBP1b, then there would be no stabilising compound (the role that ampicillin has). Additionally, there is potential that, due to the variation in structure, the conditions used for crystallisation may no longer be suitable for the ecoPBP1b/CW-019 crystal complex to grow. It should be noted that it was expected that CW-019 wouldn't bind to ecoPBP1b, but due to its similar structure to the beta-lactam core there was a possibility it would bind (similar to CENTA, as done by King *et al*. (2016)). However, due to the current lack of structures with other beta-lactams (which have been identified to bind to similar PBPs), it cannot be determined whether CW-019 is unable to bind entirely. It would be interesting to see whether the compound on its own would have inhibitory properties, as well as testing it with a clinical beta-lactam compound on a strain of bacteria with a beta-lactamase production gene. This could be carried out with preliminary MIC testing using 96 well plates.

Again, linking back to the King *et al.* study, they managed to bind a beta-lactamase substrate (CENTA) to the penicillin-binding region of ecoPBP1b. This indicates that in the right

circumstances, a beta-lactamase inhibitor may be seen to bind to the penicillin-binding region of the TP domain. Structurally, CW-019 does not actually contain a beta-lactam ring, which may prevent the binding to the TP domain. In addition, CENTA is a substrate, not an inhibitor. As beta-lactamases are designed to degrade the beta-lactam ring, it would be logical that that CENTA is capable of binding to the TP domain. Despite it not binding to the TP domain, CW-019 may still have beta-lactamase inhibitory effects, capable of being used in conjunction with beta-lactams on resistant bacterial species.

Further research would be useful into the production of novel moenomycin inhibitors, especially those containing peptide linkers. As demonstrated by this project, this is an area with significant potential for antimicrobial design. In addition, the design of such inhibitors with the ability to inhibit both beta-lactams and beta-lactamases (building on any findings from the CW-019 research) would be another point of research

4.4. Antimicrobial Resistance

The wider issues of antimicrobial resistance is an issue that is going to continue regardless of the number of antimicrobial compounds produced. As long as antibiotics have widespread use (and in many cases, improper use), bacterial species will develop resistance. There is no so-called ‘golden bullet’ when it comes to infectious diseases. Bacterial species have been prevalent since the start of life on Earth, and their ability to adapt to all kinds of environmental change has been key in their survival. Every antibiotic in clinical use has seen some level of resistance develop, including those of a ‘last line’ nature; vancomycin and erythromycin, for example. Wider initiatives are required to reduce usage of antibiotic compounds both clinically and commercially. It is understood that such changes would not be immediate, but should be effected at some level. Resistance to such changes (both from medical, veterinary and agricultural professionals as well as pharmaceutical companies) should be expected, which will limit any widespread change.

Development of novel beta-lactams is an area of research that has been up for debate, with many research groups struggling to produce compounds with significant differences and lessened resistance. Research into this area may be better focussed on producing compounds to be used in conjunction with currently clinically approved beta lactams, such as the aforementioned beta lactamase inhibitors. Whilst this would provide some longevity to our current armoury of beta lactams, this would eventually stagnate in much the same way that beta-lactam research failed to progress. Due to the multiple areas of resistance to these classes of antibiotics, this would eventually select for methods of resistance which are harder to overcome (altered PBPs, for example). In addition, it has been noted that many beta lactamase inhibitors are not particularly broad spectrum towards beta-lactamases, which limits their clinical applications (resistant infections would require genomic analysis to determine which BLase inhibitors to use in conjunction with beta lactam therapy) (Bush, 2015).

In an ideal situation, development would be best focussed on antibiotics with novel modes of action. Advances have been made in the last couple of years looking at identifying and characterising new compounds, such as the iChip, publicised in 2015 alongside the identification of a novel compound: Teixobactin (Ling *et al.* 2015).

4.5. Teixobactin; the Next Antibiotic Goldmine?

Teixobactin is known to bind to lipid II, a major precursor to peptidoglycan utilised by glycosyltransferases such as ecoPBP1b. Currently, it is only known that teixobactin prevents lipid II from binding to the transglycosylases that use it as a substrate. However it could be suggested that the teixobactin/Lipid II complex formed may additionally inhibit transglycosylases. As of yet, no such complex has been formed bound to a transglycosylase. If such a complex could be produced, it would add a second layer to the mode of action of teixobactin, improving the understanding of this novel compound.

Interest has been high in teixobactin, especially developing analogues to the naturally occurring compound. This is due to the incredibly low levels of resistance demonstrated in preliminary resistance testing. It is expected that resistance to teixobactin will eventually develop (especially in clinical settings), as has developed with all other major antibiotic compounds. However, this resistance is likely to be low level, and have a high energy cost. As mentioned, teixobactin binds to a highly conserved, essential substrate (lipid II). Alteration of this essential substrate is unlikely to occur, as it would also require the alterations to occur in the enzymes which utilise Lipid II. It is more likely that the first resistance seen will be in laboratory testing, with short-lived changes with high energy costs.

It may also be recommended that teixobactin is kept as a 'last resort' antibiotic, to prevent widespread usage and subsequent widespread resistance developing. Other methods to limit resistance development to teixobactin is to use it in conjunction with other compounds (as part of combination therapy), a method which has seen a decrease in resistance occurring.

Novel compounds (such as teixobactin) should not be hailed as new golden bullets. Instead, they should be seen for what they are; the next line of defence against an unstoppable force. These compounds will simply buy time until the following line of defence can be identified, be it another naturally occurring antibiotic, or a completely fresh style of antimicrobial entirely. Complacency, and imagined security in current compounds has brought us to this stage, and it is up to us to prevent it from occurring to this level again.

5. Conclusions

It is clear that novel antimicrobials are required in order to sustain the current standard of healthcare in most countries. The current approach to novel antimicrobial design (modifying existing families of compounds), by itself, may eventually see a decline in the numbers of useful compounds approached. Prolonging this approach may require both looking backwards (at compounds that were discarded as poor alternatives during the 'golden age' of antimicrobial discovery), as well as looking forwards, not just identifying new compounds, but designing new methods for locating them.

In the interim, more study needs to be carried out on ecoPBP1b and the role it can play as an antimicrobial target. Whilst the current understanding of ecoPBP1b is suitable for moenomycin analogues, it may be worth looking at other conditions to crystallise ecoPBP1b in complex with larger beta-lactams than ampicillin.

This study has improved upon the conditions necessary to produce useable quantities of ecoPBP1B for further studies, especially in those involving crystallisation. In addition, this project has highlighted the fact that ecoPBP1b can be used to explore novel alterations to current antimicrobials – specifically the moenomycins and beta-lactams, and improve the design of novel inhibitors such as CW-019. This will facilitate further generations of antimicrobials compounds to help in the fight against antimicrobial resistance.

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